UNIVERSITA' DEGLI STUDI DI PARMA

Dottorato di ricerca in Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti

Ciclo XXIII°

Meccanismi di controllo fisiologico e potenziali interferenti nel processo di angiogenesi follicolare

Coordinatore:
Chiar.mo Prof. Primo Mariani

Tutor:
Chiar.ma Prof.ssa Giuseppina Basini

Dottoranda: Laura Baioni

INDICE

INTRODUZIONE 1

Meccanismi di angiogenesi 4

Angiogenesi nel follicolo ovarico 10

LAVORO SPERIMENTALE 17

Funzionalità follicolare e angiogenesi: controllo fisiologico 18

Ruolo della Stanniocalcina-1 19

Ruolo della Netrina-1 23

Ruolo della Prolattina 26

MATERIALI E METODI 29

La prolattina nel follicolo ovarico suino 29

Isolamento della membrana granulosa e della teca 29

Estrazione RNA e PCR 30

Raccolta dei fluidi follicolari 32

Raccolta delle cellule della granulosa e coltura in ipossia 32

Quantificazione prolattina 33

Effetti della prolattina sulle cellule della granulosa suine 33

Steroidogenesi 34

Produzione VEGF. 34

Produzione di anione superossido (O_2^-) e perossido di idrogeno (H_2O_2) 34

Attività di detossificazione enzimatica 35

Attività di detossificazione non enzimatica 36

Effetti della prolattina sulle cellule endoteliali suine 37

Saggio biologico di angiogenesi in vitro 38

Produzione di VEGF e NO 38

Analisi statistica 39

RISULTATI 40

La prolattina nel follicolo ovarico suino 40

Espressione dei geni PRL e PRL-R 40

Quantificazione prolattina nei fluidi e nei media di coltura 42

Effetti della prolattina sulle cellule della granulosa 44

Produzione steroidi 44

Produzione VEGF 46

Produzione di O_2^- e H_2O_2 47

Attività di detossificazione enzimatica 48

Attività di detossificazione non enzimatica 49

Effetti della prolattina sulle cellule endoteliali suine 50

Saggio biologico di angiogenesi 50

Produzione VEGF e NO 53

DISCUSSIONE 55

Funzionalità follicolare e angiogenesi: interferenti esogeni 61

Effetto del Gossipolo 62

Effetto del Bisfenolo A 65

Il follicolo ovarico: un modello per lo studio di potenziali agenti terapeutici 67

BIBLIOGRAFIA 72

INTRODUZIONE

L'angiogenesi è un processo biologico che consiste nella formazione di nuovi vasi sanguigni a partire da vasi preesistenti. La formazione di nuovi vasi può avvenire mediante la gemmazione delle cellule endoteliali dei capillari preesistenti nel tessuto circostante, "sprouting" angiogenesi, o per divisione dei capillari in due o più vasi figli, "non sprouting" angiogenesi (Ucuzian e al., 2010).
La formazione di nuovi vasi è essenziale per l'organogenesi e per lo sviluppo del feto (Clapp e al., 2009), mentre dopo la nascita si verifica a livello dei siti di crescita tissutale (Wan e al., 2010). Nell' adulto, in condizioni fisiologiche, i fattori inibitori prevalgono su quelli stimolatori e, di norma, il processo non avviene (Distler e al., 2003): lo sviluppo dei capillari avviene solamente in associazione alla riparazione di ferite o fratture (Clapp e al., 2009). Un' importante eccezione è rappresentata dal tratto riproduttivo femminile, dove la neoformazione vasale è fondamentale durante le modificazioni cicliche di ovaio ed endometrio e per la funzionalità della placenta e della ghiandola mammaria durante la gravidanza (Grothius e al., 2005). La regressione dei vasi neoformati accompagna, successivamente, l'involuzione fisiologica di queste strutture.
L'angiogenesi fisiologica è un processo altamente controllato e modulato secondo le necessità del tessuto (Staton e al., 2009). La neoformazione vasale avviene attraverso una precisa successione di diverse fasi ciascuna delle quali è il risultato dell'azione coordinata di numerose molecole ad azione pro-angiogenica ed anti-angiogenica fra cui fattori di crescita solubili, citochine, proteasi, proteine di matrice e molecole d'adesione (Distler e al., 2003). Quando si verifica il cosiddetto "shift angiogenico" i fattori regolatori positivi dell'angiogenesi predominano e

le cellule endoteliali vengono attivate. L'attivazione prevede una complessa riprogrammazione funzionale delle cellule endoteliali che implica la neosintesi di alcuni geni e la repressione di altri.
Nell'ultimo decennio sono stati scoperti numerosi fattori regolatori sia pro- che anti-angiogenici ma l'effetto risultante dalla loro interazione reciproca deve essere ancora completamente chiarito.
È necessario che questo processo sia finemente regolato poiché una crescita eccessiva, una regressione aberrante (Staton e al., 2009) o uno sviluppo insufficiente dei vasi (Gonzalez e al., 2007) possono portare all'insorgenza di diverse patologie.
Alterazioni nella regolazione angiogenica sono presenti in più di 50 patologie fra cui cancro e infiammazioni croniche; inoltre, possono essere correlate a sterilità (Carmeliet e Jain 2000).
Nell'angiogenesi patologica, il rigido controllo che caratterizza l'angiogenesi fisiologica viene meno. Inoltre, i vasi neoformati raramente arrivano a maturazione e non si verifica il processo di regressione.
Pertanto, una migliore comprensione della biologia dell'angiogenesi potrebbe essere utile per individuare nuove molecole e nuovi bersagli per il trattamento delle patologie associate a questo complesso processo.
Inoltre, l'interesse allo studio del processo angiogenico è dato anche dal fatto che il successo di numerose terapie nella medicina rigenerativa necessita della capacità di controllare la formazione di una rete vasale stabile all'interno del tessuto (Francis e al., 2008).
La funzionalità ovarica è strettamente dipendente dalla formazione e dal continuo rimodellamento di una complessa rete vascolare (Robinson e al., 2009).
In particolare, lo sviluppo del follicolo ovarico rappresenta un raro esempio fisiologico di rapida crescita tissutale associata a neovascolarizzazione. Questa caratteristica distintiva rende il follicolo un importante modello per lo studio dell' intero processo angiogenico.

Inoltre, poiché la crescita del follicolo ovarico è stata paragonata a quella di un tumore solido, le conoscenze ottenute in questo sistema potrebbero essere utili anche per lo sviluppo di strategie atte a contrastare l'angiogenesi patologica che accompagna la crescita neoplastica e la formazione delle metastasi (Neeman e al., 1997). Nonostante la maggior parte dei test *in vitro* si focalizzi sulle cellule endoteliali la cui migrazione, proliferazione e differenziazione sono fondamentali per il processo angiogenico, altri tipi cellulari sono importanti per la regolazione di questo processo (Staton e al., 2009).
Ad esempio, nel follicolo ovarico un ruolo determinante nella promozione dello sviluppo vascolare è svolto dalle cellule della granulosa (Figura 1). Esse infatti sono dotate di attività angiogenica e producono la maggior parte dei fattori regolatori (Grasselli e al., 2003; Bianco e al., 2005; Fraser e al., 2006). L'attività angiogenica della granulosa è regolata da numerosi fattori presenti nel follicolo e lo stimolo principale è rappresentato dalla condizione di ipossia crescente a cui queste cellule sono sottoposte all'aumentare dello sviluppo del follicolo antrale (Bianco e al., 2005).

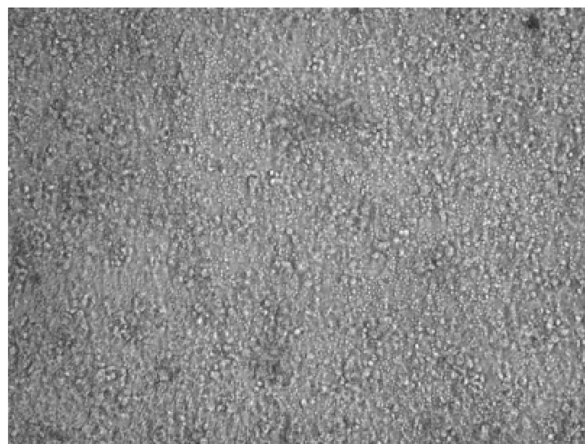


Figura 1. Cellule della granulosa in coltura. Microfotografia al microscopio a contrasto di fase.

Utilizzando come modello sperimentale il follicolo ovarico antrale suino (Figura 2), l'obiettivo della ricerca è stato quello di acquisire nuove conoscenze riguardanti:

- il ruolo di fattori proteici presenti nell'organismo quali Stanniocalcina-1, Netrina-1 e Prolattina nel controllo fisiologico della funzionalità follicolare e del processo di angiogenesi locale;
- il potenziale effetto di interferenti esogeni di varia natura chimica quali Gossipolo e Bisfenolo A sulla funzionalità follicolare e sul processo di angiogenesi;
- l'effetto angiogenico di molecole di sintesi quali analoghi del Resveratrolo e Lignani, potenzialmente utilizzabili a scopo terapeutico.

Figura 2. Follicolo ovarico antrale suino.

Meccanismi di angiogenesi

Nel sito di angiogenesi le cellule endoteliali, attivate da uno stimolo fisiologico, escono dallo stato di quiescenza e passano ad uno stato

caratterizzato da un alto indice mitotico, aumentata capacità di migrazione e di proteolisi (Ucuzian e al., 2010). Durante la crescita di un nuovo vaso si possono individuare quattro fasi (Distler e al., 2003) :

1) vasodilatazione e aumento della permeabilità
2) destabilizzazione del vaso e degradazione della matrice
3) proliferazione e migrazione delle cellule endoteliali
4) formazione del lume e stabilizzazione del vaso

Oltre all' azione di fattori pro- e anti-angiogenici, un ruolo importante nella regolazione fisiologica dell'angiogenesi è svolto anche da parametri emodinamici quali shear stress e pressione sanguigna (Distler e al., 2003; Lehoux e al., 2006). Inoltre un potente stimolo per la crescita fisiologica dei vasi sanguigni è rappresentato dall'ipossia (Figura 3).

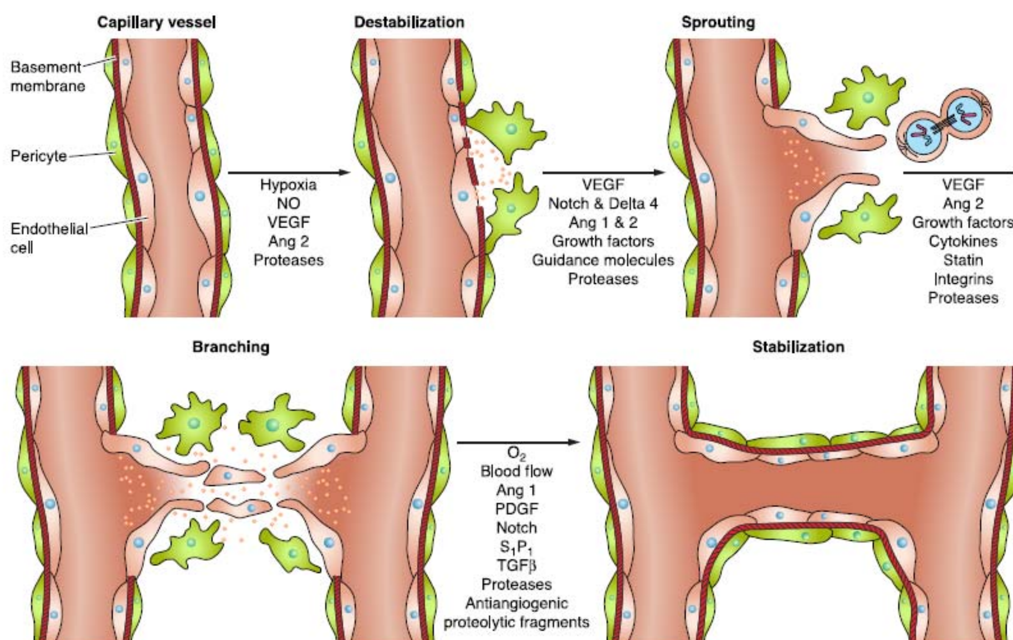


Figura 3. Fasi del processo di angiogenesi.

All'interno della cellula, il segnale ipossico è trasdotto dal fattore di trascrizione Hypoxia Inducible Factor 1α, (HIF-1α) il quale promuove rispettivamente l'up-regulation dell'espressione di diversi fattori angiogenici quali Vascular Endothelial Growth Factor (VEGF), Angiopoietina-2 e Ossido Nitrico Sintasi (NOS) (Pugh and Racliffe, 2003) e la down-regulation di fattori inibitori.
La famiglia del VEGF, il principale fattore angiogenico, comprende: VEGF-A (VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E e PGF (Placental Growth Factor). Dal punto di vista strutturale, il VEGF A è un omodimero glicoproteico presente in una varietà di isoforme diverse prodotte per splicing alternativo di un singolo gene. Fra le isoforme identificate ci sono VEGF121, VEGF145, VEGF148, VEGF162, VEGF165, VEGF 165b, VEGF183, VEGF189, VEGF206, che differiscono per composizione amminoacidica, proprietà e funzioni (Takahashi e Shibuya, 2005). I membri della famiglia del VEGF si legano a tre recettori tirosina chinasici: VEGF-R-1 (FLT-1), VEGFR-2 (KDR/Flk-1) e VEGFR-3 (FLT-4). Il VEGF inoltre interagisce con una famiglia di corecettori glicoproteici, neuropilina-1 e -2 (NRP-1 e -2) (Costa e al., 2004).
L'angiogenesi ha inizio con la vasodilatazione del vaso preesistente. L'aumento della permeabilità vascolare è promosso dall'azione di un radicale libero gassoso, l'ossido nitrico (NO) e del VEGF, il quale favorisce la distruzione delle giunzioni costituite dalle Platelet Endothelial Cell Adhesion (PECAM-1) e dalle Vascular Endothelial (VE) Caderine presenti fra cellule endoteliali e fra quest'ultime e le cellule perivascolari (Pugg e Racliffe, 2003; Armulik e al., 2005).
L'NO é sintetizzato a partire dall'amminoacido L-arginina, che viene convertito dall'enzima ossido nitrico sintasi (NOS) in citrullina, con la liberazione di NO; questa reazione richiede la presenza di cofattori come ossigeno molecolare e NADPH. Esistono tre isoforme di NOS. Due di

esse, la NOS neuronale (nNOS) e la NOS endoteliale (eNOS), sono espresse costitutivamente e per l'attivazione richiedono la presenza del sistema calcio/calmodulina, l'altra forma è inducibile (iNOS) ed è espressa in risposta all'azione delle citochine infiammatorie e di prodotti batterici ed è calcio-calmodulina indipendente (Tamanini e al., 2002).
Solamente un sottogruppo di cellule endoteliali sono selezionate per lo sprouting e rispondono ai segnali angiogenici. Le cellule attivate si possono suddividere in cellule che guidano il processo di invasione, le "tip cell" o cellule di punta, dotate di attività proteolitica e di numerosi filopodi e "stalk cell" o cellule inseguitrici; queste ultime partecipano alla formazione del nuovo vaso proliferando e provvedendo alla deposizione della membrana basale (Figura 4).

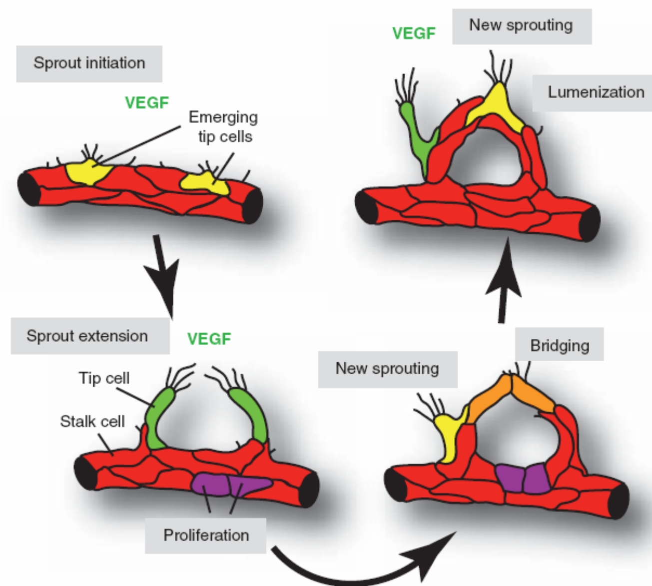


Figura 4. Sprouting angiogenesi.

I meccanismi che indirizzano le cellule endoteliali a formare nuove strutture tubulari o a rimanere nel monostrato sono ancora poco

conosciuti (Engelse e al., 2008). Componenti importanti nella regolazione di questo processo sono i recettori Notch e i ligandi corrispondenti Delta-like 4 (Gridley, 2007; Hellstrom e al., 2007).

Un ruolo importante nella regolazione dell'angiogenesi è svolto dall'interazione fra cellule endoteliali e matrice extracellulare. Le cellule endoteliali selezionate acquisiscono la capacità di invadere la membrana basale subendoteliale e la matrice extracellulare (Francis e al., 2008). Esse migrano nello spazio perivascolare utilizzando le proteine plasmatiche fuoriuscite dal vaso dilatato come matrice provvisoria. Inoltre, le cellule endoteliali rilasciano proteasi quali attivatori del plasminogeno (u-PA e t-PA), Metalloproteasi di matrice (MMP), Eparinasi, Triptasi e Catepsine, che degradano la matrice extracellulare, favorendo un ulteriore supporto alla migrazione delle cellule stesse.

La migrazione delle cellule endoteliali è guidata principalmente da VEGF, angiopoietina1 e 2 e Fibroblast Growth Factor (bFGF). Altre citochine che regolano questo processo sono il Platelet Derived Growth Factor (PDGF), l'Epidermal Growth Factor (EGF), il Trasforming Growth Factor β (TGFβ) e molecole guida quali Efrine, Semaforine e Netrine (Lamalice e al., 2007; Adams e Alitalo, 2007).

Oltre alla migrazione, l'estensione degli sprouts necessita della proliferazione delle cellule endoteliali. Questa fase è indotta principalmente da VEGF e Angiopoietina-2.

Le proteasi facilitano lo sprouting dei vasi promuovendo il rilascio di fattori angiogenici legati alla matrice (bFGF, VEGF, TGFβ) e attivando citochine angiogeniche come l'Interleuchina-1β. Inoltre, la matrice degradata favorisce l'adesione delle cellule endoteliali integrine-mediata. Il segnale, trasdotto dalle integrine quali αvβ3 e αvβ5, agisce in concerto

all'attivazione dei recettori di VEGF e bFGF nel promuovere o inibire la proliferazione delle cellule endoteliali (Serini e al., 2006).

Infine, gli sprouts si uniscono e formano strutture tubulari grazie alla fusione di vacuoli, in un primo momento intracellulari, e, successivamente, intercellulari (Kamei e al., 2006). Nella formazione del lume un ruolo importante è svolto dall'angiostatina, inibitore endogeno della angiogenesi che deriva dalla proteolisi del plasminogeno (Albini e al., 2009). È grazie alla capacità geneticamente programmata delle cellule endoteliali di formare un lume che avviene il passaggio del flusso sanguigno dal vaso preesistente al vaso neoformato. L'aumento di ossigeno, dovuto all'instaurarsi del flusso sanguigno, abbassa l'espressione locale di VEGF, e, di conseguenza, la proliferazione delle cellule endoteliali si riduce. Il reclutamento di periciti e la deposizione della membrana basale promuovono la maturazione del vaso e la sua quiescenza. Il processo di stabilizzazione è regolato da diversi fattori fra cui: Angiopoietina 1 e il suo recettore Tie 2, PDGF e PDGF receptor β, segnale Notch (Armulik e al., 2005), sfingosina-1-fosfato-1 (S1P1) e TGF-β (Allende e al., 2003; Bertolino e al., 2005). Nel controllo negativo del processo angiogenico possono inoltre contribuire le proteasi, liberando componenti della matrice ad attività antiangiogenica quali trombospondina-1 e 2 (TSP-1 e -2), canstatina, tumstatina e le forme bioattive del collagene clivato endostatina e arrestina. Altri inibitori angiogenici sono le vasoinibine, l'antitrombina III, la protrombina kringle-2, il plasminogeno kringle-5 e la vasostatina (Nyberg e al., 2005). Mentre i fattori che promuovono l'angiogenesi inibiscono l'apoptosi delle cellule endoteliali, i fattori inibitori la inducono in modo specifico favorendo il processo di regressione del vaso (Dimmeler e Zheier, 2000).

Il processo angiogenico necessita di un' elevata coordinazione fra fattori ad azione autocrina, paracrina ed endocrina. Mentre sono stati fatti

numerosi sforzi per studiare fattori la cui funzione principale è quella di regolare l'angiogenesi, il contributo fornito da sostanze pleiotropiche, come gli ormoni, risulta difficile da interpretare data la loro varietà di target e funzioni. Essi possono agire direttamente sulle cellule endoteliali o indirettamente, reclutando altri tipi cellulari a produrre fattori angiogenici quali il VEGF. In particolare, negli ultimi anni ha assunto rilievo il ruolo svolto dai membri della famiglia composta da ormone della crescita/prolattina/lattogeno placentare, il sistema renina-angiotensina e il sistema kallikreina-kinina. Questi ormoni esercitano un effetto stimolatorio sull'angiogenesi, ma possono acquisire proprietà antiangiogeniche una volta sottoposti a clivaggio proteolitico. Nell'adulto, elevati livelli circolanti di questi frammenti antiangiogenici aiutano a mantenere il sistema vascolare in uno stato di quiescenza. Tuttavia i meccanismi d'azione di questi sistemi non sono ancora stati completamente chiariti (Clapp e al., 2009).

Angiogenesi nel follicolo ovarico

Nel follicolo ovarico lo sviluppo di una rete di capillari indipendente è indispensabile per il passaggio di questa struttura dallo stadio primordiale e primario agli stadi di sviluppo successivi (Robinson e al., 2009).

Inizialmente, i capillari sono sottili e disposti in un unico strato. Lo sviluppo vascolare è limitato allo strato tecale mentre la granulosa resta avascolare per tutta la follicologenesi (Tamanini e De Ambrogi, 2004). Al momento, ci sono poche informazioni su come il follicolo recluta la sua rete vascolare. Il principale fattore angiogenico ovarico è identificato nel VEGF A. Esso è già presente nelle cellule della granulosa e nelle cellule della teca del follicolo secondario bovino (Yang e Fortune, 2007), mentre

Angiopoietina e FGF2 non appaiono in queste cellule prima dello stadio antrale (vanWezel, 1995; Hayashi e al., 2004). Non è chiaro quale sia lo stimolo per la produzione di VEGF a questo stadio dello sviluppo poiché l'HIF1 non è ancora presente (Duncan e al., 2008). Nel suino, durante lo sviluppo del follicolo preantrale, si assiste ad un aumento della densità vascolare e ad un parallelo aumento dell'espressione del VEGF mRNA sia nella teca che nella granulosa (Martelli e al., 2009).

Nell'ovaio, il processo angiogenico è coordinato all'evoluzione e all'involuzione ciclica delle strutture ivi presenti. Questo fa sí che, in questo organo, l'espressione di molti fattori angiogenici sia ormone-dipendente (Geva e Jaffe, 2004).

A questo proposito, è indicativo il fatto che il VEGF aumenta ulteriormente nelle cellule della teca e della granulosa dei follicoli quando il loro sviluppo inizia ad essere dipendente dalle gonadotropine: l'ormone follicolo stimolante, FSH e l'ormone luteinizzante, LH.

FSH e LH sono ormoni di natura glicoproteica costituiti da due subunità unite da interazioni non covalenti: la subunità α comune ad entrambi e la subunità β ormone-specifica. Nell'adulto, FSH e LH sono rilasciati in maniera intermittente dall'ipofisi anteriore con una frequenza e ampiezza delle pulsazioni variabili durante il ciclo. Questo tipo di secrezione è conseguente alla liberazione pulsatile nell'ipotalamo del fattore di rilascio delle gonadotropine, il GnRH. A seguito dell'interazione con recettori specifici, le gonadotropine danno origine a una sequenza di segnali nelle cellule bersaglio. Il legame promuove l'attivazione della subunità α della proteina G alla quale sono accoppiati. Questa proteina, a sua volta, stimola l'enzima adenilato ciclasi, causando un aumento dei livelli intracellulari di AMPc e la conseguente attivazione della proteina chinasi A (PKA) (Wood e Strauss 2002). L' AMPc regola l'espressione e l'attivazione degli enzimi deputati alla sintesi degli ormoni steroidei (Havelock e al., 2004).

In particolare, nelle cellule della granulosa, l'FSH promuove l'azione degli enzimi aromatasici (CYP19) i quali trasformano gli androgeni provenienti dalla teca in estrogeni (17β-estradiolo, estrone) (Jamnongjit e Hammes, 2006). Successivamente, a seguito del picco di LH, si verifica un rapido e consistente aumento della produzione di progesterone grazie all'azione dell'enzima 3β-idrossisteroidodeidrogenasi (3β–HSD).

L'attività degli steroidi a livello del tessuto bersaglio si esplica attraverso il legame a recettori intracellulari. Il complesso ormone-recettore viene trasferito al nucleo dove si lega al DNA, a livello del promotore di geni sensibili agli ormoni steroidei regolandone l'attività trascrizionale. Fra questi geni ci sono anche regolatori angiogenici: nel follicolo bovino è stato dimostrato che il progesterone stimola l'espressione del VEGFA mRNA (Shimzu e Miyamoto, 2007).

Inoltre, nelle cellule della granulosa, gli estrogeni promuovono la proliferazione e la differenziazione, esercitano un effetto antiatresico, regolano la formazione delle gap junction, dell'antro follicolare e la sintesi dei recettori per FSH e LH. Esistono due tipi di recettori per questi ormoni, ERα e β. Nel suino, l'espressione di ERβ è presente durante tutto lo sviluppo follicolare e aumenta in sinergia con la proliferazione della granulosa, diminuendo poi nel corpo luteo; ERα, al contrario, si osserva solo nel follicolo pre-ovulatorio e nel corpo luteo in fase precoce (Schams e Berisha, 2002).

Il progesterone favorisce il differenziamento finale delle cellule della granulosa in cellule luteiniche (Drummond, 2006) e ha un ruolo importante nel promuovere la maturazione dell'oocita e l'ovulazione (Jamnongjit e al., 2005).

L' azione delle gonadotropine e degli ormoni steroidei è indispensabile per il mantenimento della funzionalità ovarica (Findlay e al., 2009) e

fornisce un contributo importante nella regolazione dell'angiogenesi follicolare (Reisenger e al., 2007; Kim e al., 2008). L'inibizione del rilascio di gonadotropine utilizzando un antagonista del GnRH inibisce l'espressione del VEGF e l'angiogenesi nei follicoli ovulatori.

Altri ormoni peptidici stimolano l'espressione del VEGF nel follicolo. Ad esempio, IGF-1 e IGF-2 promuovono la sua espressione nelle cellule della granulosa.

Allo stadio di follicolo antrale, la vascolarizzazione è costituita da due reti di capillari concentriche, una situata direttamente sotto la membrana basale e l'altra nella teca esterna (Martelli e al., 2006). Mediante l'utilizzo della tecnica denominata "vascular corrosion casts," è stato possibile evidenziare come l' angiogenesi nella teca avvenga secondo modalità differenti durante lo sviluppo follicolare:

nel follicolo antrale precoce si osserva una gemmazione seguita dallo sprouting dei vasi, mentre, quando il follicolo antrale raggiunge il massimo sviluppo, i capillari si allungano (Jiang e al. 2003). Lo sviluppo della rete vascolare è fondamentale per la crescita del follicolo antrale. Numerosi studi hanno documentato infatti che composti antiangiogenici come il VEGF trap riducono la vascolarizzazione tecale e di conseguenza lo sviluppo follicolare (Bianco e al., 2005; Fraser e Duncan, 2009). Poiché le modalità di angiogenesi variano in associazione allo stadio di maturazione del follicolo, è probabile che in ogni fase intervengano regolatori specifici (Robinson e al., 2009). Ad esempio, è possibile che ognuna delle isoforme del VEGF A sia espressa in modo differenziale nelle varie fasi della follicologenesi.

Nel follicolo antrale, il VEGF A è principalmente localizzato nella granulosa avascolare (Greenway e al., 2005), probabilmente allo scopo di creare un gradiente che vada a stimolare lo sviluppo vascolare verso la membrana basale, massimizzando l'apporto di ossigeno, nutrienti e ormoni alle cellule della granulosa (Robinson e al., 2009). Gli effetti del

VEGF A possono essere ulteriormente modulati dall'azione coordinata con altri fattori quali Angiopoietina, FGF2 e PDGF (Greenberg e al., 2008).

La maggior parte degli studi sono rivolti all'indagine del controllo positivo dell'angiogenesi follicolare mentre i meccanismi che regolano il controllo negativo del processo sono ancora poco conosciuti. Uno dei fattori anti-angiogenici presente nel follicolo è la TSP-1. La sua concentrazione decresce all'aumentare dello sviluppo follicolare ed è massima durante l'atresia. La sua espressione è aumentata dall'LH in cellule della granulosa di ratto (Thomas e al., 2008). Per quanto riguarda gli ormoni, è stato osservato che in vitro il progesterone ha un effetto degenerativo sui vasi (Jaggers e al., 1996); un effetto inibitorio sulle cellule endoteliali è stato documentato anche per alcuni metaboliti degli estrogeni, quali 2-metossiestradiolo (Basini e al., 2006, Basini e al., 2007), 4- idrossi e 2- idrossi estradiolo (Basini e al., 2008a).

Il letto vascolare follicolare fornisce le basi sulle quali si svilupperà la rete vasale del corpo luteo. L'accumulo di fattori proangiogenici nel follicolo preovulatorio, quali VEGF e FGF, appare finalizzata a sostenere l'intensa angiogenesi che avviene dopo l'ovulazione (Robinson e al., 2009). Nel follicolo preovulatorio, lo sviluppo vascolare viene gradualmente soppiantato dal processo di maturazione vascolare e questo è evidenziato dall'incremento del rapporto angiopoietina1/angiopoietina2 che si verifica a questo stadio nel bovino (Hayashi e al., 2004). Il picco di LH induce l'espressione di numerosi geni che inducono l'ovulazione ed è stato visto che la gonadotropina può avere un effetto diretto sull'angiogenesi (Robinson e al., 2007).

Nel periodo periovulatorio si instaura una condizione di iperemia e aumento del flusso sanguigno (Acosta e al., 2003). Questo è probabilmente dovuto ad un aumento della produzione di NO dovuta ad una aumentata espressione degli enzimi ossido nitrico sintasi

endoteliale (eNOS) ed inducibile (iNOS) nei vasi tecali. Probabilmente, tale effetto è promosso dall'azione dell'estradiolo, considerato un rapido promotore dell'eNOS nelle cellule endoteliali (Kim e al., 2008). L'aumento di permeabilità è favorito anche dal VEGF. Nel suino, l'HIF1 è sovraespresso nel follicolo periovulatorio e nel follicolo subito dopo l'ovulazione. È possibile che LH induca direttamente l'espressione di HIF1 (Robinson e al., 2009). Il picco di LH induce anche la sovraespressione di numerose proteasi che promuovono la degradazione della membrana basale e l'invasione dei vasi nella granulosa avascolare (Berisha e al., 2008). La rottura della membrana favorisce la liberazione di fattori angiogenici sequestrati nella membrana stessa e la differenziazione delle cellule della granulosa in cellule luteiniche (Robinson e al., 2009). Inoltre, l'aumento dell'attività proteolitica favorisce la degradazione della matrice extracellulare che circonda i vasi esistenti. Una proteasi importante nel follicolo è la Disintegrin and Metalloproteinase with a TSP type 1 motif (ADAMTS1). Essa cliva i proteoglicani di matrice quali versicano e aggrecano e pro-collagene. È indotta dalle gonadotropine, probabilmente mediante HIF-1 (Kim e al., 2009).

Diversi studi hanno attribuito un ruolo importante nella trasduzione del segnale angiogenico a molecole appartenenti al gruppo delle specie reattive dell'ossigeno (ROS) quali il perossido d'idrogeno (H_2O_2) e l'anione superossido (O_2^-) (Basini e al., 2004). Queste molecole sono presenti in tutte le cellule come sottoprodotti del metabolismo aerobico (Valko e al., 2007); una fonte importante nelle cellule della granulosa è inoltre rappresentata dall'elevata attività metabolica e steroidogenica (Basini e al., 2008b). Mentre, a concentrazioni moderate, essi possono fungere da molecole segnale, il loro accumulo può, invece, causare danni alle strutture cellulari. Questo ha portato gli organismi a sviluppare una serie di meccanismi di difesa di tipo enzimatico e non

enzimatico che agiscono sui ROS regolandone i livelli intracellulari (Cadenas, 1997). Gli antiossidanti enzimatici comprendono la superossido dismutasi (SOD), la glutatione perossidasi (GPx) e la catalasi (CAT). La SOD agisce sull' O_2- convertendolo in H_2O_2, la quale deve essere poi degradata dalla catalasi e/o dalla perossidasi. Gli antiossidanti non enzimatici comprendono differenti sostanze fra cui l'acido ascorbico (vitamina C), la vitamina E, il glutatione, i carotenoidi, i flavonoidi, il selenio, lo zinco, la taurina e l'ipotaurina. Il follicolo preovulatorio è dotato di potenti difese antiossidanti, che contrastano l'elevata produzione di ROS (Agarwal e al., 2005) proteggendo l'oocita e garantendo un maggiore successo riproduttivo.

LAVORO SPERIMENTALE

- **FUNZIONALITÀ FOLLICOLARE E ANGIOGENESI:**

  - *Controllo fisiologico*
    - Ruolo della STANNIOCALCINA-1
    - Ruolo della NETRINA-1
    - Ruolo della PROLATTINA

  - *Interferenti esogeni*
    - Effetto del GOSSIPOLO
    - Effetto del BISFENOLO A

- **IL FOLLICOLO OVARICO: UN MODELLO PER LO STUDIO DI POTENZIALI AGENTI TERAPEUTICI**

Funzionalità follicolare e angiogenesi: controllo fisiologico

Ruolo della Stanniocalcina-1

La stanniocalcina1 (STC1) è un ormone glicoproteico coinvolto nel mantenimento dell'omeostasi del calcio e del fosfato nei pesci teleostei. La sua presenza è stata più recentemente dimostrata anche nei mammiferi. Il primo omologo della STC nei mammiferi, STC1, è stato isolato indipendentemente in due laboratori: in un caso durante studi eseguiti per identificare geni coinvolti nella proliferazione cellulare (Chang e al., 1995) e nell'altro durante un sequenziamento random del cDNA di polmone umano (Olsen e al., 1996). Questa scoperta ha creato un grande interesse circa il possibile ruolo di questo ormone nei mammiferi. La STC1 umana è costituita da 247 aminoacidi e mostra una sorprendente omologia con quella del pesce (circa 50%); le estremità carbossi-terminali, tuttavia, sono molto diverse: questo indica che l'attività biologica risiede nel core e nel dominio N-terminale. In particolare, un residuo di 11 cisteine è completamente conservato, così come il sito di glicosilazione (Niu e al., 2000). La presenza di una cisteina non accoppiata in posizione 170 (169 nel pesce) consente la formazione di un ponte disolfuro e la formazione di un omodimero di 50-kDa (STC-50) che rappresenta la forma dell'ormone maggiormente diffusa (Zhang e al., 1998; Gerritsen e Wagner, 2005).

Il ruolo biologico della STC1 non è stato ancora completamente chiarito, ma i dati disponibili evidenziano un suo coinvolgimento in diversi processi fisiologici ed anche in diversi eventi patologici. Numerose evidenze (Zhang e al., 2000; Stasko e Wagner, 2001; Chakraborty e al., 2007) concordano con il fatto che queste diverse azioni potrebbero essere mediate dall'effetto della STC sul trasporto locale di calcio e fosfato, anche se numerosi altri effetti non sono correlati con l'omeostasi minerale (Deol e al., 2000). Mediante Northern Blot è stata dimostrata in diversi mammiferi la presenza del trascritto a livello di

rene, surrene, cuore, polmone, timo e muscolo scheletrico: l' espressione quantitativamente più elevata è stata osservata a livello ovarico (Varghese e al., 1998).

Diversi laboratori hanno osservato una up-regulation della STC in cellule endoteliali durante l'angiogenesi tumorale (Gerritsen e al., 2002; Liu e al., 2003; Wary e al., 2003). È stato inoltre dimostrato che l'rhSTC inibisce la migrazione delle cellule endoteliali indotta dall'Hepatocyte Growth Factor (HGF), mentre la risposta chemotattica al VEGF e all'FGF sembra non essere modulata dal cotrattamento con la STC. La STC1 potrebbe avere pertanto un ruolo selettivo e modulatorio nell'angiogenesi fungendo, probabilmente, da "segnale di stop" o da fattore stabilizzante e contribuendo alla maturazione dei vasi sanguigni neoformati (Zlot e al., 2003).

La STC è in grado di indurre, inoltre, cambiamenti significativi nell' espressione di numerosi geni nelle cellule endoteliali in coltura. Questo fatto, associato alla sua presenza sulla superficie apicale di cellule endoteliali in vivo, presuppone un coinvolgimento di questo ormone nella regolazione di numerosi aspetti della funzione endoteliale (Chakraborty e al., 2007).

Lo stato ormonale è in grado di influenzare l'espressione della STC1 nell'ovaio (Ishibashi e Imai, 2000). La sua espressione è regolata dall'LH e dagli alti livelli di progesterone.

In un lavoro precedente è stata documentata la capacità di influire sulla produzione del VEGF da parte delle cellule della granulosa suine *in vitro* (Basini e al., 2009). Questo dato lascia ipotizzare un ruolo nella regolazione dell'angiogenesi follicolare. Tuttavia il ruolo svolto dalla STC1 nello sviluppo del follicolo ovarico non è ancora stato completamente chiarito e, al momento, non ci sono dati relativi alla sua presenza nella specie suina.

Nel lavoro "Expression and localization of stanniocalcin 1 in swine ovary" (Allegato A, reperibile nella versione definitiva al link: http://www.sciencedirect.com/science?_ob=MImg&_imagekey=B6WG0-4Y0T912—1—9&_cdi=6808&_user=606283&_pii=S0016648009004407&_origin=search&_coverDate=04%2F01%2F2010&_sk=998339997&view=c&wchp=dGLbVtz—zSkWA&md5=4fc9f5cc7a3e1cac0ffba64f083f37d0&ie=/sdarticle.pdf; doi.10.1016/j-ygcen.2009.12.013) è stata valutata la presenza dell'ormone in sezioni istologiche di ovaio mediante indagine immunoistochimica. Parallelamente, è stata ricercata l'espressione del gene STC1, mediante RT-PCR, nelle componenti cellulari (strato tecale, cellule della granulosa e oocita) di follicoli antrali a diverso grado di sviluppo. Un altro obiettivo è stato quello di valutare se la STC1 è presente nel fluido follicolare e la eventuale produzione da cellule della granulosa in coltura. Inoltre, è stato valutato un potenziale effetto dell' ipossia. La ricerca della proteina nei fluidi e nei media condizionati è stata effettuata attraverso l'impiego di un saggio RIA, grazie alla collaborazione del Prof. Wagner dell'Università del Western Ontario. I risultati ottenuti hanno dimostrato che la STC1 è presente nell'ovaio suino. A livello del follicolo antrale, il gene è espresso in tutte le componenti cellulari e l'entità dell'espressione nella teca e nella granulosa è dipendente dal grado di sviluppo del follicolo stesso. La proteina è presente nel fluido follicolare e viene prodotta dalle cellule della granulosa. Inoltre la condizione di deprivazione di ossigeno stimola tale produzione. Questi dati suggeriscono un ruolo potenziale della STC1 nella regolazione della fisiologia ovarica.

Nel secondo studio "Stanniocalcin 1 is a potential physiological modulator of steroidogenesis in the swine ovarian follicle" (Allegato B, disponibile nella versione definitiva al link:
http://www.springerlink.com/content/n81536639483956g/fulltext.pdf ; doi: 10.1007/s11259-009-9252-1), si è proceduto alla valutazione

dell'effetto dell'ormone su uno dei principali parameri funzionali di una coltura primaria di cellule della granulosa: l'attività steroidogenica. La determinazione dei livelli di estradiolo 17 β (E2) e progesterone (P4) è stata eseguita con un dosaggio RIA. L'ormone è in grado di influire sulla produzione da parte delle cellule della granulosa di entrambi gli steroidi (E2: effetto inibitorio, $p<0,001$; P4: effetto stimolatorio, $p<0,05$). I risultati ottenuti dimostrano che la STC1 è un potenziale regolatore fisiologico del processo di steroidogenesi del follicolo antrale suino.
Infine, nel lavoro "Stanniocalcin 1 affects redox status of swine granulosa cells", sottoposto alla rivista Regulatory Peptides (Allegato C), sono stati valutati gli effetti della STC1 su altri parametri fondamentali per la funzione fisiologica della granulosa e del follicolo: proliferazione, produzione dei ROS e attività dei principali sistemi enzimatici e non enzimatici deputati alla loro detossificazione. La proteina non ha effetto sulla proliferazione della granulosa. Al contrario, modula la produzione dei ROS (O_2^- effetto stimolatorio, $p<0,001$; H_2O_2, effetto inibitorio, $p<0,001$) e l'attività degli enzimi perossidasi (effetto inibitorio, $p<0,05$) e catalasi (effetto stimolatorio, $p<0,01$) mentre non influisce sull'attività della SOD. La presenza dell'ormone, inoltre, potenzia la risposta dei sistemi non enzimatici cellulari indagata mediante FRAP assay ($p<0,05$). I risultati dimostrano che l'azione fisiologica della STC1 nel follicolo potrebbe essere mediata dalla capacità della proteina di modulare lo stato ossido-riduttivo della granulosa.

Ruolo della Netrina-1

Le netrine sono proteine secrete che regolano la migrazione dei neuroni e guidano gli assoni lungo un percorso definito durante la formazione del sistema nervoso centrale (Kennedy e al. 1994; Serafini e al., 1996). Precisamente, rappresentano segnali bifunzionali in quanto svolgono una funzione attrattrice o repulsiva a seconda che avvenga il legame alla famiglia recettoriale DCC (Delete in Colorectal Carcinomas) o UNC-5 (Uncoordinated) (Kennedy e al., 1994). Nei vertebrati esistono diversi tipi di netrine. Nel pollo, sono state identificate la Netrina-1 e -2, mentre nel topo, nel ratto e nell'uomo sono state individuate la Netrina-1, -3, -4 e –G (Wang e al., 1999; Koch e al., 2000; Miyashita e al., 2005).

Le netrine e i relativi recettori sono presenti anche in altri organi, come pancreas, polmoni e ghiandola mammaria: questa evidenza ha suggerito l'ipotesi di un più ampio coinvolgimento di queste molecole nei processi morfogenetici (Engelkamp, 2002). Inoltre, recentemente è stato evidenziato un possibile ruolo delle netrine nella guida delle cellule endoteliali durante la formazione dei vasi sanguigni (Wilson e al., 2006). La Netrina-1 risulta coinvolta in vari processi cellulari quali adesione, migrazione e proliferazione (Yurchenco e al., 2004). La struttura di tale proteina vede all'estremità N-terminale la presenza di regioni omologhe ai domini V e VI delle laminine. Infatti, il dominio V contiene 3 frammenti ripetuti simili all'Epidermal Growth Factor (EGF), sia in termini di numero che di tipologia di residui cisteinici. Al contrario, il dominio C-terminale non mostra omologie con quello delle laminine, ma presenta numerosi residui basici, con sequenze BXBB o BXB (B = residui basici) (Kappler e al., 2000), (Figura 5).

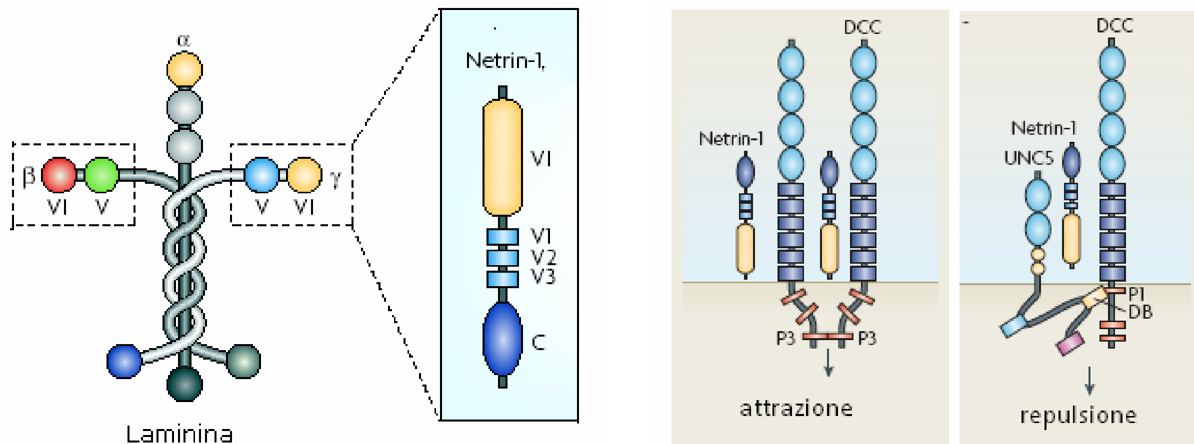


Figura 5. Struttura della NETRINA-1 e recettori.

Allo stato attuale, le uniche evidenze sperimentali sulla presenza della proteina a livello ovarico sono riferite alle sole cellule della granulosa suine (Maeda e al., 2008); tuttavia, il ruolo di questa proteina a livello ovarico non è ancora stato completamente chiarito.

Inoltre, Lu e collaboratori (2004) hanno dimostrato che la Netrina-1 svolge un ruolo repulsivo durante la crescita della rete vascolare a seguito del legame con il recettore UNC5B. Al contrario, Park e collaboratori in uno studio dello stesso anno riportano un effetto promotore della migrazione e della proliferazione endoteliale.

Per valutare se questo fattore possa contribuire alla regolazione fisiologica dei processi di follicologenesi e angiogenesi ovarica è stato condotto uno studio *in vitro*.

I risultati ottenuti sono presentati nei lavori “The axonal guidance factor netrin-1 as a potential modulator of swine follicular function” (Allegato D, reperibile nella versione definitiva al link

http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6T3G-50R2342—1&_user=606283&_coverDate=01%2F01%2F2011&_rdoc=1&_fmt=high&_orig=search&_origin=search&_sort=d&_docanchor=&view=c&_acct=C000031458&_version=1&_urlVersion=0&_userid=606283&md5=085dcf0fb3ab5aba646e29aa30ae8251&searchtype=a;
doi: 10.1016/j.mce.2010.08.001) e "Netrin-1: just an axon-guidance factor?" (Allegato E, reperibile nella versione definitiva al link http://www.springerlink.com/content/l7431136h0n52503/fulltext.pdf; doi 10.1007/s11259-010-9366-5).

Per indagare la presenza della Netrina-1 nell'ovaio suino, si è proceduto alla ricerca della proteina in sezioni istologiche mediante indagine immunoistochimica.

Successivamente, è stata ricercata l'espressione del gene Net-1 mediante RT-PCR nelle cellule della granulosa e della teca isolate da follicoli antrali. Inoltre, è stata indagata la presenza della proteina nel fluido follicolare mediante immunoblotting. Per l'analisi funzionale sono stati valutati gli effetti della Netrina-1, gentilmente fornita dalla Prof.ssa Hinck dell'Università della California, (5, 30, e 100 ng/ml) su una coltura primaria di cellule della granulosa e su una linea di cellule endoteliali aortiche suine immortalizzate (AOC). I risultati ottenuti hanno dimostrato che la Netrina-1 è espressa in entrambe le componenti cellulari del follicolo ovarico suino e che la proteina è presente nel fluido follicolare. Inoltre, la proteina ha avuto un effetto inibitorio ($p<0,05$) sulla crescita delle AOC in un saggio biologico di angiogenesi *in vitro*. Tale effetto potrebbe essere mediato dalla capacità della proteina di modulare la produzione dei fattori angiogenici VEGF e NO ($p<0,01$).

Inoltre, la Netrina-1 ha modulato in modo significativo ($p<0,05$) vitalità e attività steroidogenica della granulosa. Questi dati indicano la Netrina 1 quale potenziale regolatore di diversi aspetti della fisiologia follicolare.

Ruolo della Prolattina

La prolattina (PRL) è un ormone polipeptidico sintetizzato e secreto da cellule specializzate dell'ipofisi anteriore dette lattotrope (Freeman e al., 2000). Il termine "prolattina" deriva dalla funzione per la quale è stata caratterizzata per la prima volta: l'induzione della lattazione. Nel corso degli anni, tuttavia, le funzioni attribuite alla prolattina sono aumentate fino ad arrivare ad identificarne oltre 300 (Grattan e Kokay, 2008).

La prolattina è presente in tutti i vertebrati e il suo cDNA è stato isolato e sequenziato in diverse specie. La proteina è costituita da una singola catena aminoacidica con tre ponti disolfuro fra sei residui di cisteina (Cooke e al., 1981).

Nel ratto e nel topo la prolattina è costituita da 197 aminoacidi, mentre nella pecora, nel suino, nel bovino e nell'uomo da 199 con una massa molecolare di circa 23000 Da. La struttura tridimensionale della proteina è definita da 4 α–eliche antiparallele (Figura 6).

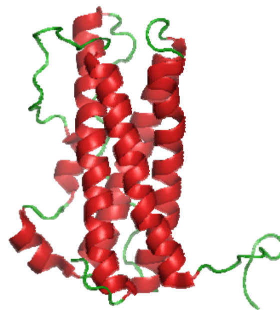


Figura 6. Struttura molecolare della prolattina.

Anche se la forma maggiormente diffusa è quella di 23 kDa, varianti sono state caratterizzate in diverse specie. Le varianti possono derivare dallo splicing alternativo del trascritto primario, dal clivaggio proteolitico e da modificazioni post-traduzionali della catena aminoacidica quali fosforilazione e glicosilazione (Freeman e al., 2000).

Oltre ad essere sintetizzata e secreta dalle cellule lattotrope dell'ipofisi anteriore, l'espressione del gene PRL è stata dimostrata in diverse regioni del cervello, nella decidua, nel miometrio, nelle ghiandole lacrimali, nel timo, nella milza, nei linfociti circolanti, nelle cellule linfoidi del midollo osseo, nelle cellule epiteliali mammarie e tumorali, nei fibroblasti cutanei e nelle ghiandole sudoripare. La prolattina è inoltre presente in numerosi fluidi corporei oltre al siero quali: liquido cerebrospinale, liquido amniotico, lacrime, latte, fluido follicolare e sudore.

Mentre la prolattina ipofisaria agisce secondo la classica via endocrina, la prolattina extraipofisaria agisce in modo diretto fungendo da fattore di crescita, neurotrasmettitore o immunomodulatore.

La prolattina prodotta localmente può agire sulle cellule adiacenti (azione paracrina) o sulle stesse cellule che l'hanno prodotta (azione autocrina) (Bole-Feysot e al., 1998).

Il recettore della prolattina (PRL-R) appartiene alla classe 1 della superfamiglia dei recettori delle citochine (Kelly e al., 1991) ed è costituito da un dominio extracellulare un dominio transmembrana e un dominio intracellulare. La trascrizione del gene è regolata da tre differenti promotori tessuto-specifici. Il promotore I specifico per le gonadi, il promotore II per il fegato, e il promotore III presente in diversi tipi di tessuto. Diverse isoforme del recettore sono state descritte in diversi tipi di tessuto (Davis e Linzer, 1989; Ali e al., 1991). Esse derivano dallo splicing alternativo di un unico gene (Binart e al., 2010). Le isoforme differiscono per la lunghezza e composizione del dominio

citoplasmatico mentre il dominio extracellulare è identico (Leseur e al., 1991). Le tre isoforme principali identificate nel ratto sono: forma corta (291 aa), intermedia (393 aa) e lunga (591 aa). In seguito al legame con la prolattina, il recettore va incontro a dimerizzazione (Bole-Feysot e al., 1998). L'attivazione ligando-mediata del recettore porta alla fosforilazione delle tirosine di numerose proteine cellulari, compreso il recettore stesso.

La presenza di entrambi i recettori della prolattina a livello ovarico (Binart e al., 2010) è indicativa del fatto che questo ormone ha un ruolo importante nella regolazione della fisiologia dell'organo. In particolare, l'azione dell'ormone appare fondamentale per la funzionalità del corpo luteo. Nel ratto l'azione "luteotropica" è caratterizzata da un incremento della produzione di progesterone. A conferma di questo, il knock out del recettore per la prolattina porta alla perdita della funzione luteinica ed alla sterilità. Tuttavia, gli effetti della prolattina sul corpo luteo dipendono dalla specie e dalla fase del ciclo estrale. Oltre al corpo luteo, il recettore per la prolattina media numerose funzioni nelle cellule della granulosa e negli oociti (Bole-Feysot e al., 1998). A tale proposito è stato osservato che l'aggiunta di prolattina al medium di maturazione degli oociti favorisce lo sviluppo di embrioni organizzati.

Un altro aspetto importante ancora poco conosciuto è quello della produzione locale ovarica della prolattina. L'espressione del gene è stata evidenziata nell' ovaio di suino (Einspanier e al., 1986), bovino (Erdmann e al., 2007), uomo (Phelps e al., 2003). Tuttavia il significato biologico di questa produzione locale non è stato ancora completamente chiarito.

La prolattina *in vivo* promuove l'angiogenesi e regola il tono vascolare anche se è stato dimostrato che la sua azione può dipendere dal microambiente e dal tipo di cellule endoteliali sulle quali agisce. È stato dimostrato che le cellule endoteliali di diverse specie e distretti vascolari

producono prolattina (Corbacho e al., 2000; Ochoa e al., 2001). La proteina prodotta potrebbe agire direttamente legandosi al suo recettore specifico presente sulle cellule endoteliali stesse (Ricken e al., 2006). Inoltre, è stato osservato che la prolattina promuove il rilascio di VEGF da parte di macrofagi e l'espressione del gene in cellule Nb2 e cellule epiteliali mammarie di topo (HC11). Pertanto, essa potrebbe anche promuovere l'angiogenesi indirettamente agendo su cellule diverse dalle endoteliali (Clapp e al., 2008).

Inoltre è stato dimostrato che la prolattina modula l'attività della eNOS: l'infusione di prolattina in suini causa vasocostrizione del letto vascolare locale (Molinari e al., 2007).

Basandoci su queste evidenze sperimentali, abbiamo cercato di acquisire nuove conoscenze sulla presenza locale della prolattina nel follicolo ovarico antrale e su una sua potenziale azione in questo distretto. È stata inoltre indagata una eventuale capacità dell'ormone di modulare la funzionalità delle cellule endoteliali.

MATERIALI E METODI

La prolattina nel follicolo ovarico suino

Isolamento della membrana granulosa e della teca

I follicoli ovarici sono stati suddivisi in tre gruppi in funzione del loro diametro: piccoli (< 3 mm), medi (3-5 mm) e grandi (> 5 mm) (Basini e al., 2008). Dopo isolamento del singolo follicolo dal parenchima ovarico, si è proceduto in ambiente sterile, previa eliminazione della sierosa, alla separazione dei due strati parietali. Brevemente, la parete follicolare è stata perforata e rovesciata; le cellule della granulosa sono state

raccolte per gravità in una Petri con PBS sterile e si è proceduto all'eliminazione dell'oocita tramite aspirazione. La sospensione cellulare così ottenuta è stata pellettata mediante centrifugazione a 300 x g per 10 minuti e si è quindi proceduto all'estrazione dell'RNA totale. La parete tecale è stata immediatamente congelata in azoto liquido e quindi sottoposta ad omogeneizzazione mediante triturazione con pestello in mortaio di porcellana. L'operazione è stata eseguita mantenendo il tessuto in azoto liquido.

Estrazione RNA e PCR

L'estrazione dell'RNA è stata effettuata utilizzando il kit NucleoSpin® RNA II (Macherey-Nagel GmbH, Duren, Germania) seguendo la procedura indicata dal produttore. L'RNA estratto è stato eluito in 60 µl di H_2O DEPC (Dietilpirocarbonato 0,1%, RNase-free) e sottoposto a quantificazione spettrofotometrica (Gene Quant Pro, Amersham Bioscences, Freiburg, Germania). La retrotrascrizione dell'RNA totale in cDNA è stata effettuata mediante kit High-Capacity cDNA Reverse Trascription (Applied Biosystem INC, Foster City, CA, USA).

Il cDNA ottenuto è stato successivamente amplificato mediante l'esecuzione di una nested-PCR, variante della PCR in cui viene effettuata una doppia amplificazione del gene di interesse mediante l'utilizzo, in successione, di due coppie di primer complementari al PRL mRNA suino.

La prima amplificazione è stata condotta per 40 cicli. Dopo un incubazione iniziale a 95°C per 5 minuti, le temperature e i tempi per denaturazione, annealing ed estensione sono stati rispettivamente 95 °C per 30 secondi, 55°C per 30 secondi e 72°C per 30 secondi. A questo è seguita un estensione finale di 10 minuti a 72°C. I primer esterni (MWG Biotec, Ebersberg, Germania) utilizzati sono stati: forward 5'-

ACAGGGTCGTCACAGAAAGG-3’ e reverse 5’-TCCTGCATACCCCTCACTTC-3’ e la dimensione dell’amplificato ottenuto è di 401pb.

Gli amplificati della PRL ottenuti sono stati sottoposti ad una seconda PCR utilizzando una coppia di primer interni: forward 5’-CCTACTGCTGCTGGTGTCAA-3’ e reverse 5’-TTGGGCTTGCTCTTTGTCTT-3’ il cui prodotto è di 268 pb. Per l’amplificazione sono stati utilizzati lo stesso numero di cicli, le stesse temperature e gli stessi tempi descritti precedentemente.

Inoltre, il cDNA ottenuto è stato impiegato in una PCR per rilevare la presenza del trascritto del recettore della prolattina (PRL-R). I primer utilizzati sono stati: forward 5’-ATCTGGTTGGCTCACACTCC-3’ e reverse 5’-CACGAAGATCCACATGGTTG-3’ che danno un amplificato di 244 pb.

La reazione è stata condotta per 35 cicli. Dopo un incubazione iniziale a 95°C per 5 minuti, le temperature e i tempi per denaturazione, annealing ed estensione sono stati rispettivamente 95 °C per 30 secondi, 55°C per 30 secondi e 72°C per 30 secondi. A questo è seguita un estensione finale di 10 minuti a 72°C.

Gli amplificati ottenuti sono stati sottoposti ad elettroforesi in gel d’agarosio all’1,5% addizionato del colorante Gel Red (Biotium, Hayward, CA). Per l’acquisizione delle immagini è stata utilizzata una fotocamera digitale Power Shot A610 Canon (Canon Italia s.p.a., Milano).

Per controllare l’integrità del cDNA e la correttezza della reazione è stata anche analizzata l’espressione di un gene housekeeping, la β–actina porcina. I primer utilizzati sono stati: pACTIN sense (5’- GAG ACC TTC AAC ACG CCG-3’) e pACTIN antisense (5’-GGA AGG TGG ACA GCG AGG-3’) che danno un amplificato pari a 685 bp.

Raccolta dei fluidi follicolari

I fluidi follicolari sono stati raccolti mediante aspirazione dalle tre classi follicolari descritte in precedenza. Gli aspirati follicolari sono stati centrifugati per 10 minuti a 300 g per separare il fluido dalla frazione cellulare. I supernatanti sono stati congelati a -20°C fino al momento del dosaggio.

Raccolta delle cellule della granulosa e coltura in ipossia

Le cellule della granulosa sono state raccolte da follicoli grandi (> 5 mm) mediante aspirazione del liquido follicolare utilizzando un ago da 26 gauge ed immediatamente sospese nel medium di coltura (MC) contenente eparina (50 UI/ml). Si è quindi proceduto alla centrifugazione a 300 x g per 10 minuti al fine di ottenere un pellet, successivamente risospeso con cloruro di ammonio allo 0,9 % ed incubato a 37°C per un minuto per lisare gli eritrociti presenti.

Dopo ulteriore centrifugazione a 300 x g per 10 minuti, è stato eseguito il conteggio delle cellule presenti previa valutazione della loro vitalità tramite trypan blue (0,4%).

Le cellule sono state poste in piastre da 24 pozzetti a densità di 10^6/pozzetto in 1 ml di MC costituito da DMEM/Ham's F-12 supplementato con bicarbonato di sodio (2,2 mg/ml) albumina sierica bovina (BSA, 0,1%), selenio (5 ng/ml), transferrina (5 μg/ml), penicillina (100 UI/ml), streptomicina (100 μg/ml), amfotericina B (2,5 μg/ml).

Dopo la piastratura le cellule sono state incubate a 37°C in atmosfera umidificata (5% CO_2) per 24 ore e successivamente sottoposte per 18h a normossia (19% O_2), ipossia parziale (5% O_2), e ipossia totale (1% O_2) L'ipossia parziale è stata realizzata mediante Anaereocult ® C mini mentre quella totale utilizzando Anaereocult ® A mini (Merck KgaA, Darmstad, Germany). Entrambi i sistemi si basano sull'utilizzo di un

sacchetto costituito da terra silicea, ferro in polvere, acido citrico e carbonato di sodio, i quali, dopo essere stati bagnati, fissano chimicamente l'ossigeno generando un'atmosfera ipossica e anossica.

Quantificazione prolattina

La quantificazione della prolattina nei fluidi follicolari e nei media di coltura della granulosa è stata effettuata mediante metodica ELISA. Brevemente, un anticorpo secondario ovino (1:500) viene fatto adsorbire alle pareti del pozzetto. Successivamente, per saturare tutti i possibili siti di legame aspecifico si esegue un incubazione con albumina sierica bovina (BSA). Terminata questa operazione, si aggiungono il campione e l'anticorpo primario diretto contro la prolattina porcina (1:50000). Dopo incubazione, la piastra viene decantata e viene aggiunta prolattina porcina precedentemente biotinilata (20 ng/pozzetto). L'antigene va a legarsi a tutti i siti di legame dell'anticorpo rimasti liberi dopo l'incubazione con il campione. Si aggiungono la streptavidina coniugata con la perossidasi ed il substrato cromogeno ABTS. La lettura è avvenuta a 405 nm mediante Multilabel Counter Victor^3 (Perkin Elmer, Waltham, Massachusetts, USA).

Effetti della Prolattina sulle cellule della granulosa suine

La raccolta delle cellule della granulosa è stata allestita come precedentemente descritto. In base al tipo di esperimento, le cellule sono state piastrate a densità variabile. Dopo piastratura, le cellule sono state trattate con prolattina porcina ricombinante (fornita dalla Prof.ssa Farmer, Agricolture and Agri-food Canada, Dairy and Swine

Research and Development Centre, Quebec, Canada; Farmer e Palin, 2005) alle concentrazioni 0,1-1 e 10 nM e successivamente incubate a 37°C in atmosfera umidificata (5%CO_2) per 48 ore.

Steroidogenesi

Le cellule della granulosa sono state seminate in piastre da 96 pozzetti alla concentrazione di 10^4 cellule/200 μl di MC addizionato con androstenedione (28ng/ml). Dopo 48 ore di incubazione si è proceduto alla raccolta dei surnatanti, congelati a -20°C fino all'analisi del contenuto di estradiolo 17β e di progesterone, effettuata mediante RIA (Grasselli e al., 1993).

Produzione VEGF

Le cellule della granulosa sono state coltivate in piastre da 24 pozzetti alla concentrazione di 10^6/1 ml MC. Il VEGF prodotto in presenza e assenza di prolattina è stato dosato dopo 48 ore mediante ELISA (Quantikine, R&D System, Minneapolis, MN, USA); questo saggio, sviluppato per il VEGF umano, è stato validato per la specie suina (Barboni e al., 2000). La reazione colorimetrica che si sviluppa è letta a una lunghezza d'onda di 450 nm con l'ausilio di Multilabel Counter Victor[3]

Produzione di anione superossido (O_2^-) e perossido di idrogeno (H_2O_2).

La produzione di O_2^- è stata valutata mediante saggio colorimetrico con reagente WST-1 (4-3-4-Iodophenyl-2-4nitrophenyl-2H-5-terazolio-1,3-benzene; Roche, Penzberg, Germania). L'anione superossido presente riduce il sale terazolico a formazano, composto solubile in acqua quantificabile mediante spettrofotometria.

Brevemente, in piastre da 96 pozzetti sono state seminate 10^5 cellule/200 μl di MC e trattate come precedentemente descritto. Durante le ultime 4 ore di incubazione sono stati addizionati 20 μl/pozzetto di reagente WST-1. Al termine di questo periodo è stata quantificata l'assorbanza leggendo in doppia lunghezza d'onda (450 contro 620 nm) mediante spettrofotometro Multilabel Counter Victor[3].
La produzione di H_2O_2 è stata misurata utilizzando "Amplex Red Hydrogen Peroxide Assay Kit" (Molecular Probes, PoortGebouw, Olanda), che valuta la produzione di resorufina, derivante dalla reazione tra l'Amplex Red e l' H_2O_2 presente nel campione. Brevemente, 2 x 10^5 cellule sono state seminate in 200 μl di MC in piastre da 96 pozzetti e incubate come precedentemente descritto.
Al termine le cellule sono state sottoposte a centrifugazione per 10 minuti a 400 x g e, dopo eliminazione del surnatante, si è proceduto alla loro lisi mediante aggiunta di Triton 0,5% + PMSF (floruro di fenilmetansulfonile) in PBS (200 μl/pozzetto) ed incubazione in ghiaccio per 30 minuti. Sui lisati è stato poi eseguito il dosaggio, utilizzando una curva standard con punti compresi tra 0,39 e 25 μM; la lettura è avvenuta a 450 nm con Multilabel Counter Victor[3].

Attività di detossificazione enzimatica.
I procedimenti per realizzare le colture ed i lisati cellulari sono gli stessi descritti nel precedente paragrafo.
L'attività della SOD è stata determinata nei lisati cellulari, utilizzando il "SOD Assay Kit" (Dojindo Molecular Technologies, Kumamoto, Giappone). Il test colorimetrico misura quantitativamente il formazano prodotto dalla reazione tra il sale di tetrazolio (WST-1) e l'anione superossido, prodotto da una xantina ossidasi esogena. L'assorbanza è

stata determinata in doppia lunghezza d'onda, 450 contro 620 nm, mediante Multilabel Counter Victor[3].
L'attività della catalasi è stata misurata usando "Amplex Red Catalase Assay Kit" (Molecular Probes, PoortGebouw, Olanda), che si basa sulla produzione di resorufina derivante dalla reazione tra H_2O_2, fornita in eccesso e il reagente Amplex Red in presenza della perossidasi. I lisati cellulari sono stati utilizzati previa diluizione 1:20 e letti contro una curva standard con valori compresi tra 62,5 e 1000 mU/ml. L'assorbanza è stata valutata ad una lunghezza d'onda di 450 nm usando il lettore per micropiastre Multilabel Counter Victor[3].
L'attività della perossidasi è stata valutata tramite "Amplex Red Peroxidase Assay Kit" (Molecular Probes, PoortGebouw, Olanda), che quantifica la produzione di resorufina derivante dalla reazione tra H_2O_2 fornita in eccesso e il reagente Amplex Red in presenza della perossidasi presente nel campione. I lisati cellulari sono stati letti contro una curva standard con valori compresi tra 0,078 e 10 mU/ml. L'assorbanza è stata determinata ad una lunghezza d'onda di 540 nm mediante Multilabel Counter Victor[3].

Attività di detossificazione non enzimatica

Il metodo FRAP (Ferric Reducing Ability of Plasma) valuta la capacità che gli antiossidanti presenti nel campione hanno di ridurre in condizioni di pH acido il complesso Fe^{+++}/ trifiridiltriazina.
La riduzione dello ione ferrico (Fe^{+++}) a ione ferroso (Fe^{++}) avviene secondo una reazione colorimetrica valutabile tramite spettrofotometro a 593 nm (Liu e al., 1982; Benzie e Strain, 1996) rispetto ad una curva di taratura allestita con concentrazioni note di solfato ferroso. Brevemente, 40 µl di lisato cellulare sono stati addizionati a 260 µl di reagente FRAP in triplicato in piastra da 96 wells. Il reagente FRAP è

stato ottenuto miscelando 25 ml di buffer acetato, 2,5 ml di soluzione TPTZ (2,4,6-tri-(2-piridil)-s-triazina 10 mM in HCl 40 mM) e 2,5 ml di soluzione di $FeCl_3$-$6H_2O$. Dopo 30 minuti di incubazione a 37°C si è proceduto alla lettura con spettrofotometro Multilabel Counter Victor[3].

Effetti della Prolattina sulle cellule endoteliali suine

La linea immortalizzata di cellule endoteliali di aorta suina (AOC) (Figura 7) utilizzata negli esperimenti che seguono è stata gentilmente fornita dal Prof. Josè Yelamos (Hospital Universitario Virgen de la Arrixaca, El Palmar, 30120 Murcia, Spain). Le cellule sono state tutte utilizzate al 12° o 13° passaggio e coltivate in medium M199 addizionato con bicarbonato di sodio (2.2 mg/ml), penicillina (100 UI/ml), streptomicina (100 μg/ml) e amfotericina B (2.5 μg/ml) e con il 20% di FBS.

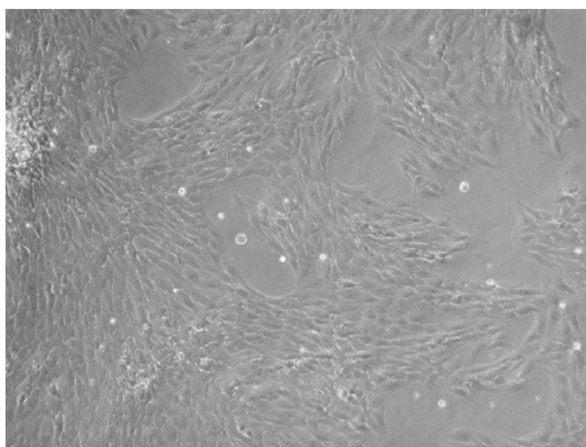


Figura 7. AOC in coltura. Microfotografia al microscopio a contrasto di fase.

Saggio biologico di angiogenesi in vitro

12,5 mg di microsferette cytodex-3 (MC) rivestite di collagene sono state idratate per tre ore a 37°C con 1,5 ml di PBS sterile. Dopo due lavaggi, le MC sono state poste in una fiaschetta insieme a cellule endoteliali aortiche suine immortalizzate (AOC) ($5 X 10^5$) in 5 ml di M199 al 2% di FBS. La fiaschetta è stata incubata per 24 ore a 37° in modo da consentire l'adesione delle AOC alle MC. Per la preparazione del gel di fibrina, le MC ricoperte da AOC (20 μl) sono state poste in piastre da 12 pozzetti contenenti una soluzione di fibrinogeno (1 mg/ml, pH 7,6) a cui è stata aggiunta la trombina (5 UI/ml). Le piastre così allestite sono state lasciate per 30 minuti a 37°C per favorire la polimerizzazione della fibrina. Successivamente, è stato aggiunto 1 ml di medium M199 in ogni pozzetto ed è stato lasciato equilibrare per 60 minuti. Dopo un cambio di medium, i gel di fibrina sono stati trattati con prolattina 0,1-1 e 10 nM ed incubati per 48 e 96 ore a 37°C al 5% di CO_2. Al fine di determinare l'area di sviluppo vascolare, al termine dell' incubazione sono state acquisite immagini fotografiche. Per quantificare la proliferazione delle AOC su gel di fibrina, ci si è avvalsi del programma Scion Image 4.02 (Scion Corporation, MA, USA, http://rsb.info.nih.gov/nih-image/), che consente di calcolare l'area occupata dalle cellule endoteliali espressa come numero di pixel.

Produzione di VEGF e NO

$2x10^5$ AOC/ 200μl sono state coltivate in piastre da 96 pozzetti con M199 e 20% FBS. Le cellule sono state trattate con la prolattina e dopo 48 ore i sopranatanti sono stati raccolti per il dosaggio di VEGF e NO. Il dosaggio del VEGF è stato eseguito come precedentemente descritto per le cellule della granulosa;

il contenuto di ossido nitrico è stato determinato mediante test di Griess, preparando due soluzioni:

- A (1% sulfanilamide, 5% acido fosforico)
- B (0.1% N-[naftil] eilenediamina diidrocloruro)

Si allestisce quindi una curva standard costituita da 8 punti, compresi tra 25 μM e 0,39 μM. A 100 μl di campione o a 100 μl di ogni punto della curva sono addizionati 50 μl di soluzione A e 50 μl di soluzione B. Dopo 10 minuti si sviluppa una reazione colorimetrica misurata a 540nm con l'ausilio del Multilabel Counter Victor[3] .

Analisi statistica

I dati vengono presentati come valore medio ± ES (errore standard). Ogni esperimento è stato ripetuto almeno cinque volte; nelle colture cellulari sono stati realizzati sei replicati per ogni trattamento. In tutti gli esperimenti, le differenze statistiche sono state calcolate mediante ANOVA utilizzando il software Statgraphics (STSC Inc., Rockville, MD, USA). Quando è stata rilevata una differenza statisticamente significativa ($p<0,05$), le medie sono state sottoposte al test F di Scheffè per confronti multipli.

RISULTATI

La prolattina nel follicolo ovarico suino

Espressione dei geni PRL e PRL-R

L'espressione del gene PRL è stata evidenziata in entrambe le componenti cellulari del follicolo, teca e granulosa, solamente nei follicoli antrali a più elevato grado di sviluppo (Figura 8).

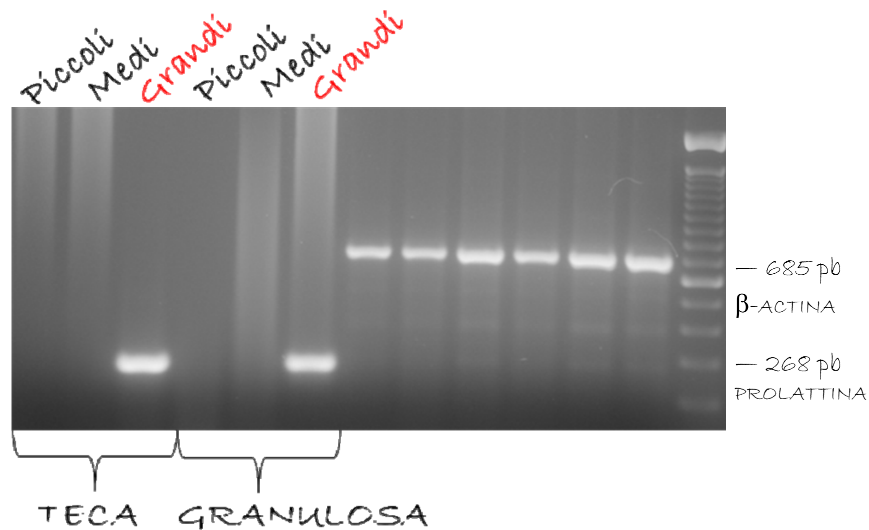


Figura 8. Espressione della prolattina e della β actina nella teca e nelle cellule della granulosa isolate da follicoli antrali di suino a diverso grado di sviluppo.

L'espressione del gene PRL-R è stata evidenziata in tutte le classi di follicoli in entrambe le componenti cellulari (Figura 9).

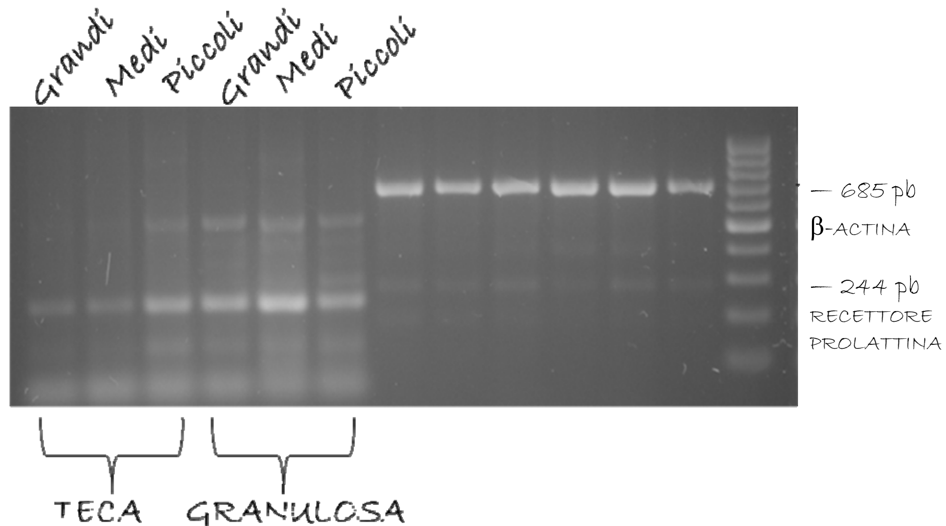


Figura 9. Espressione del recettore della prolattina e della β actina nella teca e nelle cellule della granulosa isolate da follicoli antrali di suino a diverso grado di sviluppo.

Quantificazione Prolattina nei fluidi e nei media di coltura

La prolattina è presente nel fluido follicolare suino in quantità crescente in relazione al grado di sviluppo follicolare ($p<0,05$) (Figura 10).ed è prodotta dalle cellule della granulosa risultando significativamente stimolata ($p<0,05$) dalle condizioni di ipossia (Figura 11).

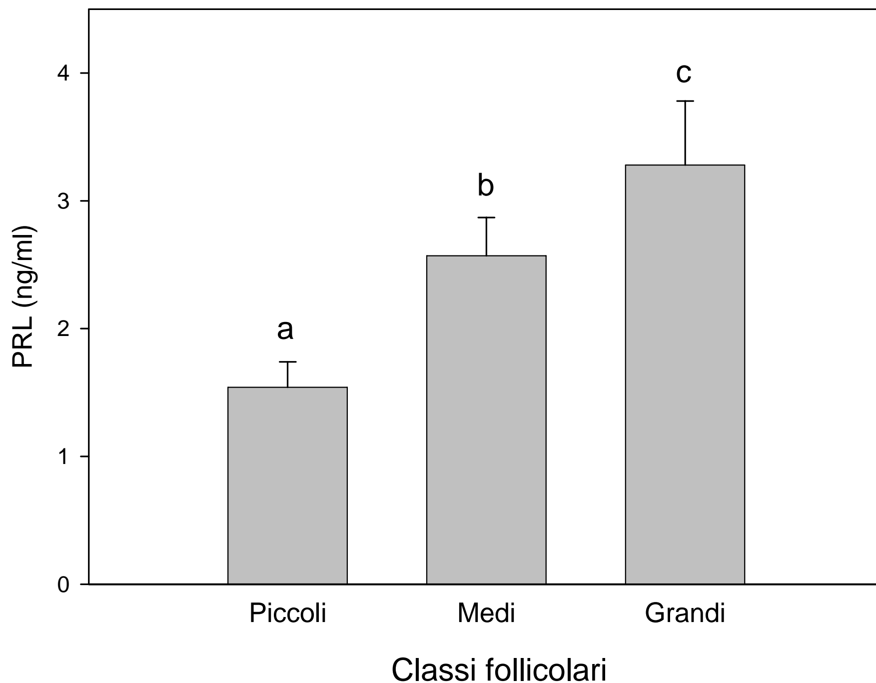


Figura 10. Contenuto di prolattina nei fluidi follicolari prelevati da follicoli piccoli, medi e grandi. I dati sono espressi come media ± ES. Lettere diverse indicano una differenza significativa ($p < 0,05$) .

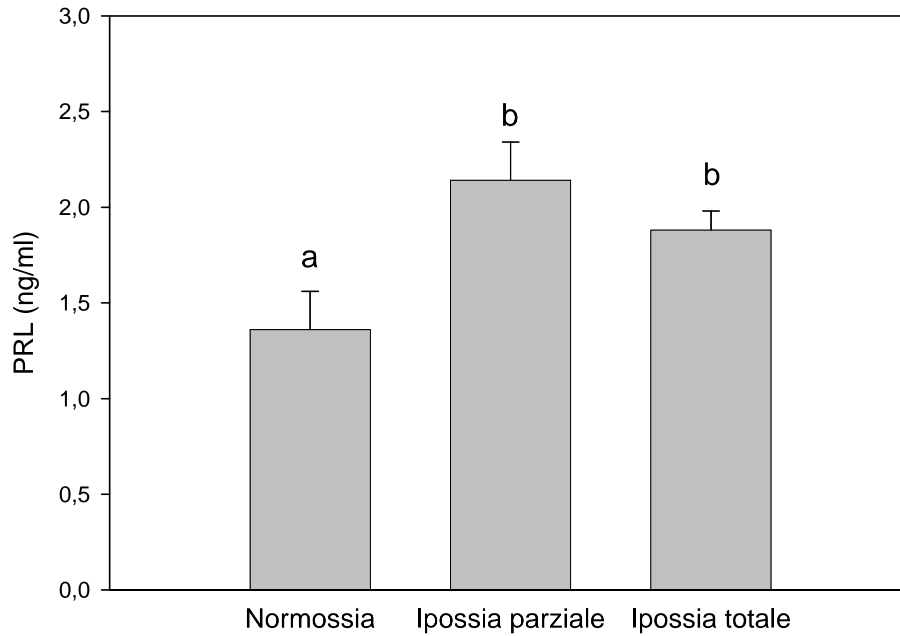


Figura 11. Effetto dell'ipossia parziale e totale sulla produzione di prolattina da parte delle cellule della granulosa suine. I dati sono espressi come media ± ES. Lettere diverse indicano una differenza significativa ($p < 0,05$).

Effetti della Prolattina sulle cellule della granulosa suine

Produzione steroidi
La prolattina ha modulato in modo significativo l'attività steroidogenica della granulosa causando, alle concentrazioni 1 e 10 nM, un effetto inibitorio sulla produzione di entrambi gli steroidi, E2 ($p<0,05$) (Figura 12) e P4 ($p<0,01$) (Figura 13).

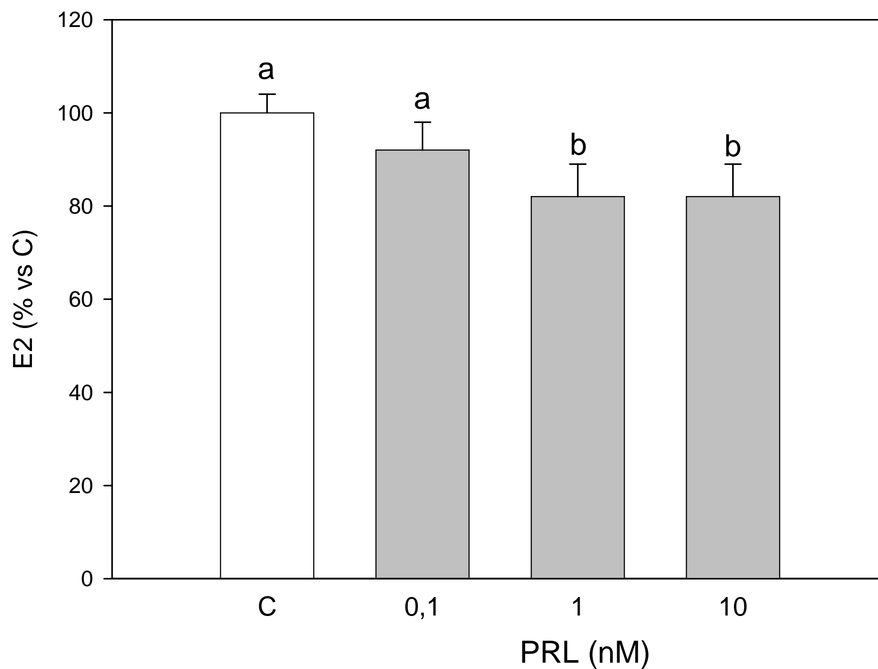


Figura 12. Produzione di E2 da parte delle cellule della granulosa suine a seguito del trattamento con prolattina. I dati sono espressi come media ± ES. Lettere diverse indicano una differenza significativa ($p < 0,05$).

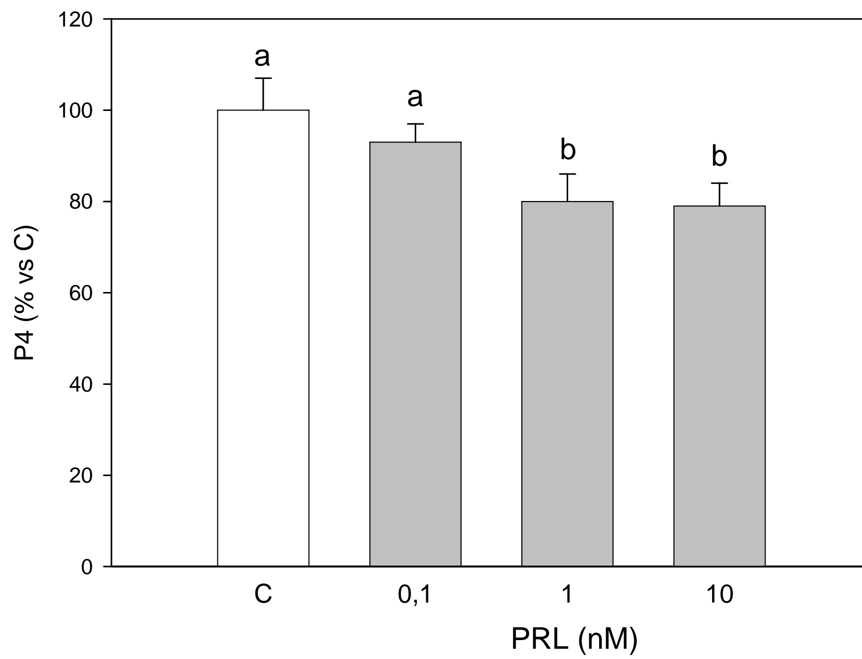


Figura 13. Produzione di P4 da parte delle cellule della granulosa a seguito del trattamento con prolattina.

I dati sono espressi come media ± ES. Lettere diverse indicano una differenza significativa ($p < 0,01$).

Produzione di VEGF

La prolattina ha modulato la produzione del VEGF. In particolare, è stato osservato un aumento ($p<0,05$) della produzione del fattore angiogenico in presenza delle concentrazioni maggiori (Figura 14).

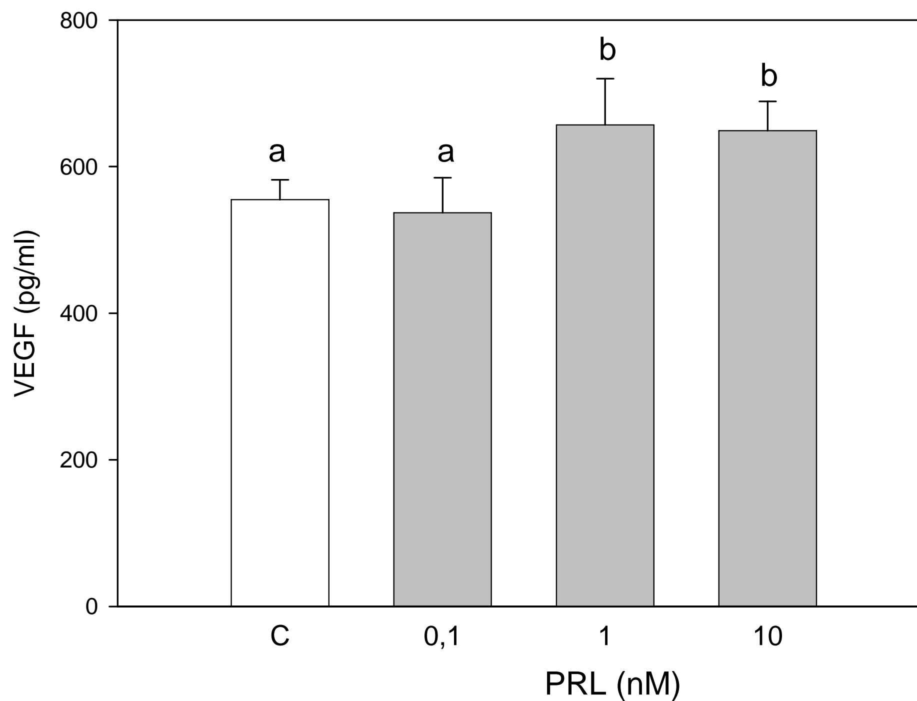


Figura 14. Produzione di VEGF da parte delle cellule della granulosa suine a seguito del trattamento con prolattina.

I dati sono espressi come media ± ES. Lettere diverse indicano una differenza significativa ($p < 0,05$).

Produzione di O_2^- e H_2O_2.

La produzione di O_2^- ha subito un decremento significativo ($p<0,001$) in presenza del trattamento 1nM (Figura 15). La produzione di H_2O_2.è stata inibita ($p<0,01$) dalla concentrazione maggiore, 10nM (Figura 16).

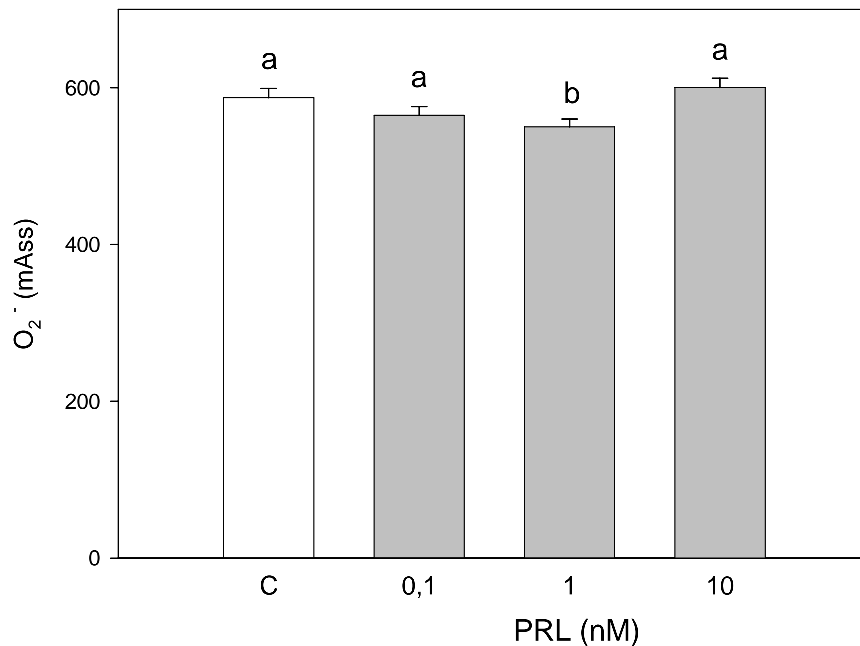


Figura 15. Effetto della prolattina sulla produzione di O_2^- da parte delle cellule della granulosa suina.

I dati sono espressi come media ± ES. Lettere diverse indicano una differenza significativa ($p < 0,001$).

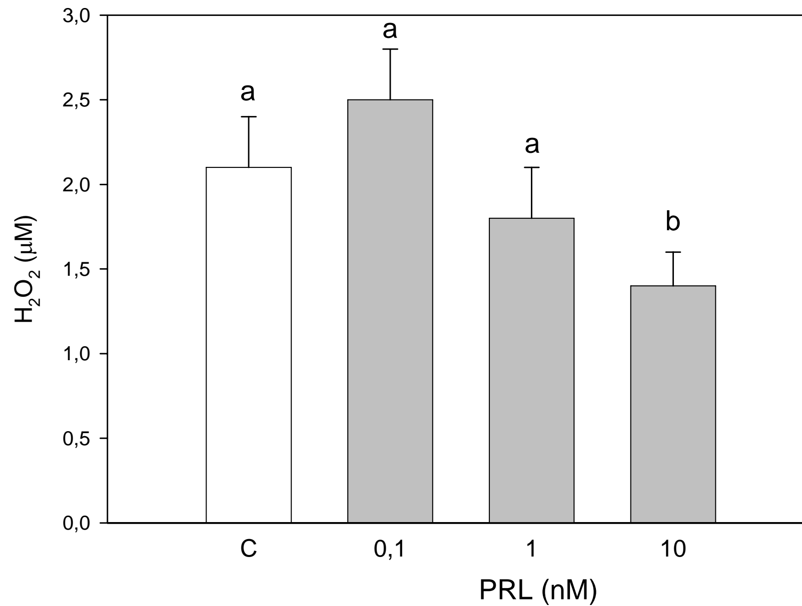


Figura 16. Effetto della prolattina sulla produzione di H_2O_2 da parte delle cellule della granulosa suina.
I dati sono espressi come media ± ES. Lettere diverse indicano una differenza significativa ($p < 0,01$).

Attività di detossificazione enzimatica

La prolattina non ha influito in modo significativo sull'attività degli enzimi di detossificazione, SOD, perossidasi e catalasi.

Attività di detossificazione non enzimatica

La capacità di detossificazione non enzimatica della granulosa, indagata mediante FRAP assay, ha avuto un incremento significativo ($p<0,001$) in presenza di prolattina 10nM (Figura 17) .

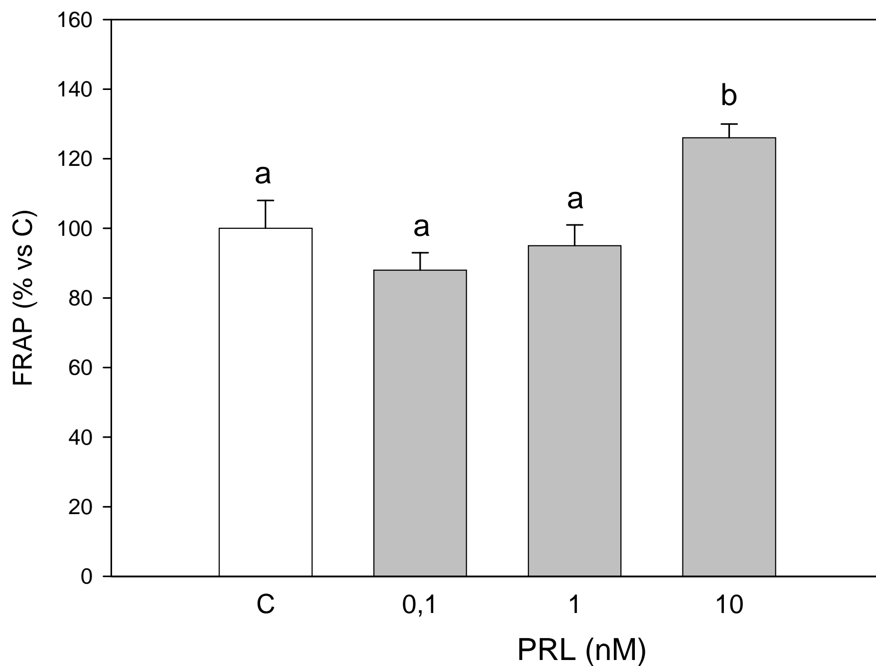


Figura 17. Effetto della prolattina sulla capacità antiossidante non enzimatica in cellule della granulosa.

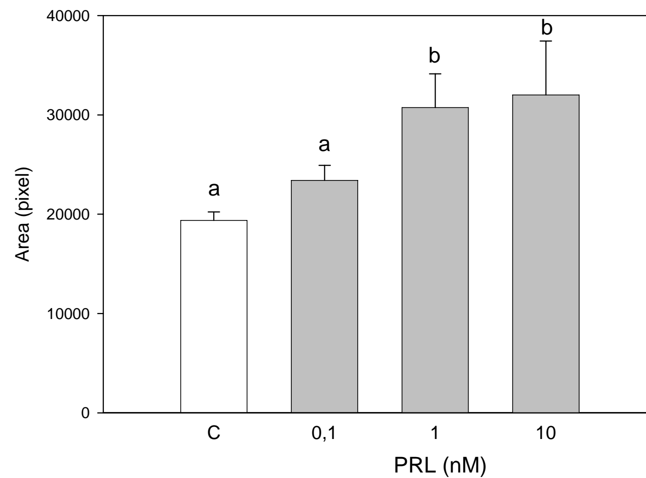
I dati sono espressi come media ± ES. Lettere diverse indicano una differenza significativa ($p < 0,001$).

Effetti della Prolattina sulle cellule endoteliali suine

Saggio biologico di angiogenesi

Dopo 48h è stata osservata una stimolazione significativa della crescita endoteliale ($p<0,001$) indotta dai trattamenti 1 e 10nM (Figura 18). Dopo 96h, è stato osservato un effetto stimolatorio ($p<0,05$) sulla crescita delle AOC in gel di fibrina anche in presenza del trattamento a concentrazione inferiore (Figura 19).

A)



B)

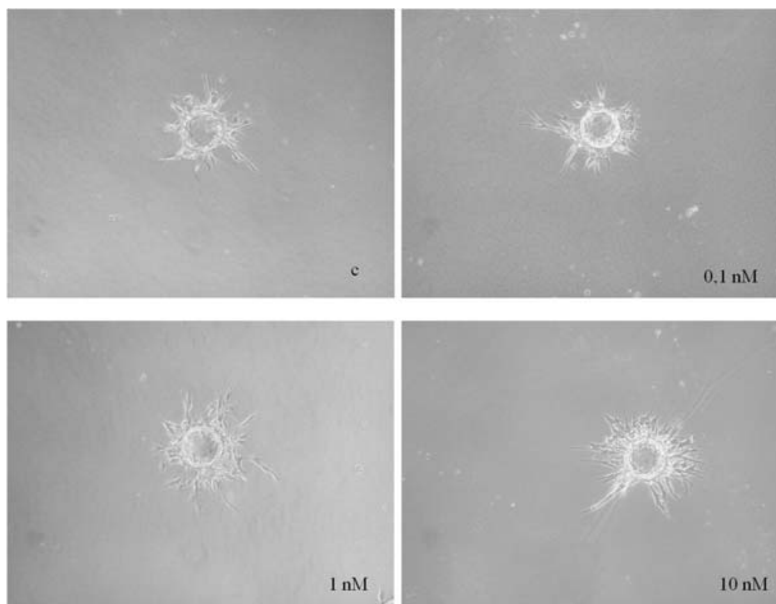
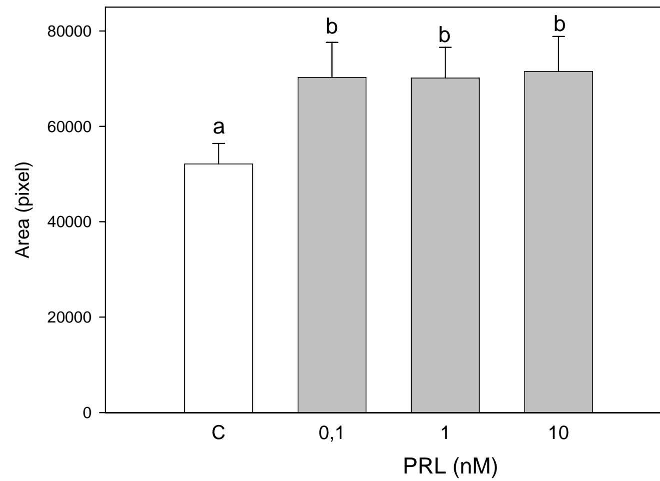


Figura 18. A) Effetto della prolattina sulla crescita delle AOC in gel di fibrina dopo 48 ore di trattamento. Lettere differenti indicano una differenza significativa (p<0,001).

B) Immagine rappresentativa al microscopio a contrasto di fase che mostra l'effetto della prolattina sulla crescita delle AOC dopo 48 ore.

A)



B)

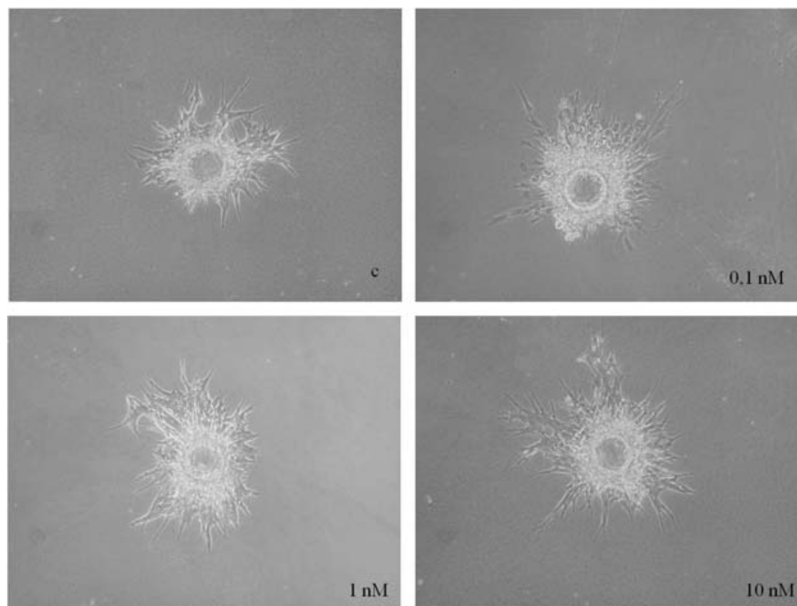


Figura 19. Effetto della prolattina sulla crescita delle AOC in gel di fibrina dopo 96 ore di trattamento. Lettere diverse indicano una differenza significativa (p<0,05).

B) Immagine rappresentativa al microscopio a contrasto di fase che mostra l'effetto della prolattina sulla crescita delle AOC dopo 96 ore.

Produzione VEGF e NO

Il trattamento con prolattina ha influito in modo significativo sulla produzione di VEGF e NO: in particolare, la quantità di VEGF prodotto aumenta ($p<0,001$) in presenza dei trattamenti 1 e 10 nM (Figura 20) mentre la quantità di NO prodotta subisce un decremento significativo ($p<0,05$) a tutte e tre le concentrazioni testate (Figura 21).

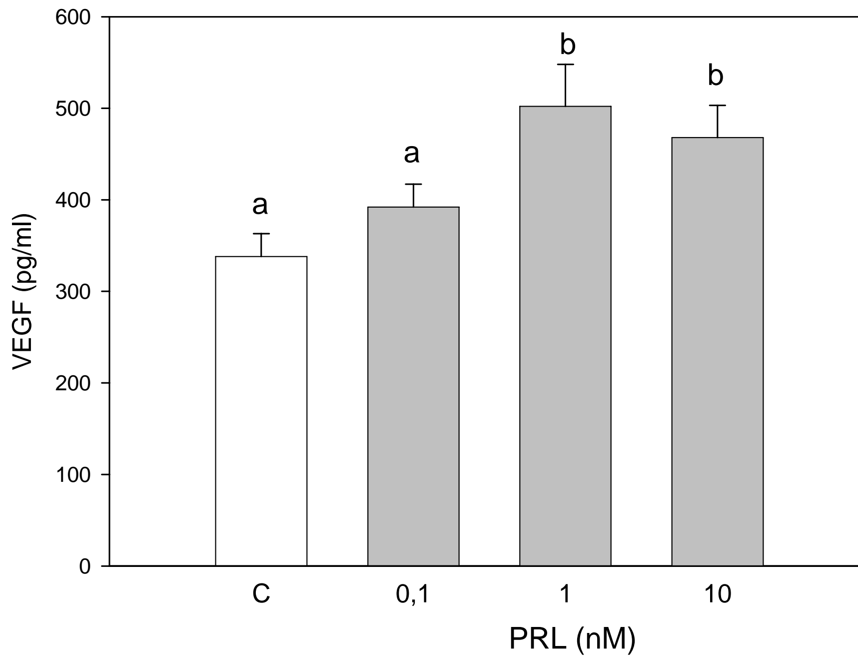


Figura 20. Produzione di VEGF nelle AOC a seguito del trattamento con prolattina.

I dati sono espressi come media ± ES. Lettere diverse indicano una differenza significativa ($p < 0,001$).

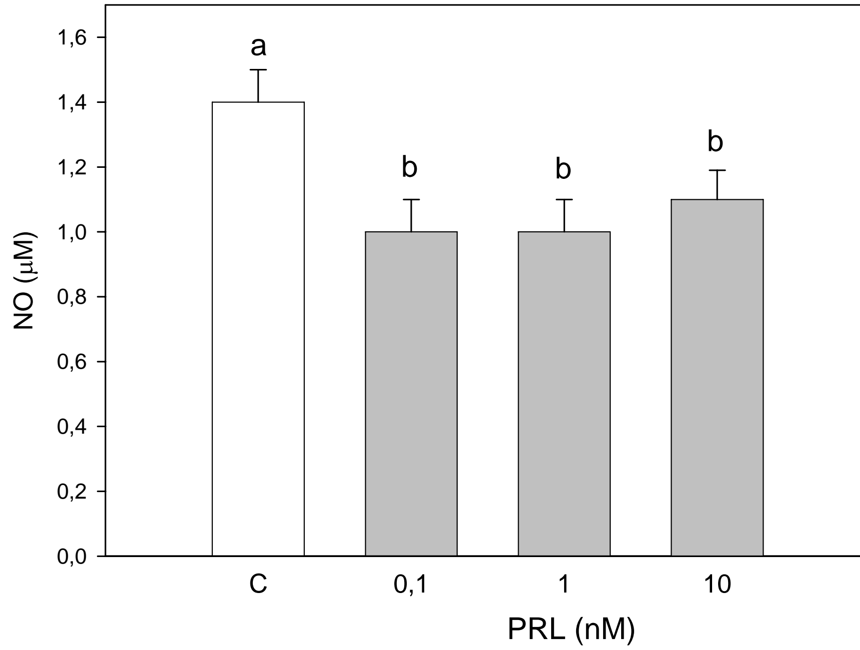


Figura 21. Effetto del trattamento con la prolattina sulla sintesi di NO da parte delle AOC. I dati sono espressi come media ± ES. Lettere diverse indicano una differenza significativa (p<0,05).

DISCUSSIONE

I dati ottenuti in questo studio qualificano il follicolo antrale suino come un sito di sintesi extraipofisaria della prolattina. In particolare, entrambe le componenti cellulari del follicolo, strato tecale e cellule della granulosa, rappresentano le sedi potenziali della sintesi locale.
La presenza del trascritto nelle cellule della granulosa conferma quanto osservato per la prima volta nel suino da Einspanier e collaboratori nel 1986, mentre l'espressione a livello della teca nella stessa specie non era mai stata evidenziata in precedenza.
La necessità di impiegare una nested PCR per rilevare l'espressione del gene è indicativa del fatto che i livelli di espressione in questo distretto sono bassi, come osservato precedentemente in cellule della granulosa umane (Phelps e al., 2003). Tuttavia, tale produzione potrebbe essere sufficiente ad esercitare un attività biologica significativa a livello locale. Inoltre, è importante sottolineare che la trascrizione del gene ha raggiunto livelli evidenziabili solamente quando il follicolo antrale raggiunge un più marcato grado di sviluppo ed entra nello stadio preovulatorio. Appare dunque significativo come la presenza del trascritto risultati strettamente dipendente dallo stadio di sviluppo follicolare; la produzione locale di prolattina potrebbe dunque intervenire nella regolazione dei processi di maturazione terminale del follicolo e nelle fasi successive, ovulazione e formazione del corpo luteo, struttura dove è stata rilevata sintesi locale di prolattina (Erdmann e al., 2007). Nell'uomo, i recettori per la prolattina non sono presenti nel follicolo antrale precoce ma solo nelle fasi di sviluppo successive, a conferma del ruolo dell'ormone negli stadi di sviluppo avanzati del follicolo antrale e negli eventi successivi (Perks e al., 2003).
Al contrario, nel suino abbiamo rilevato la presenza del trascritto del recettore anche nei follicoli antrali piccoli sia nello strato tecale che

nelle cellule della granulosa. Pertanto, in questa specie la prolattina potrebbe agire già a partire dalle prime fasi di sviluppo del follicolo antrale. In questa fase, poiché la proteina non viene prodotta a livello locale, i recettori potrebbero essere attivati dalla prolattina presente nel fluido follicolare, evidentemente proveniente da altre fonti: abbiamo infatti rilevato la presenza dell'ormone nei fluidi di tutte tre le classi follicolari. In particolare, i dati ottenuti mostrano come i livelli maggiori siano presenti nei fluidi provenienti dai follicoli antrali a più elevato grado di sviluppo. Questo era stato osservato anche nel bovino (Henderson e al., 1982).

Un' ulteriore conferma della produzione locale della prolattina è data dal fatto che la presenza della proteina è stata rilevata nei sopranatanti delle cellule della granulosa isolate da follicoli antrali grandi.

Mentre il significato biologico della produzione locale non è stato ancora ben caratterizzato, l'azione della prolattina ipofisaria sulla fisiologia ovarica è nota da tempo. La presenza della prolattina è essenziale per la funzionalità del sistema riproduttivo femminile: topi PRL^{--} non sono fertili e presentano un ciclo estrale irregolare. Al contrario, elevati livelli di prolattina hanno un effetto inibitorio sulla funzionalità ovarica poiché questo ormone inibisce la secrezione delle gonadotropine (Tay e al., 1992); in particolare, l'ormone riduce la frequenza e l'ampiezza della secrezione pulsatile di LH ed inibisce il rilascio di GnRH nel circolo portale (Grattan e Kokay, 2008).

Studi *in vitro* dimostrano che la prolattina agisce direttamente sulle cellule della granulosa potenziando o inibendo la biosintesi degli steroidi e modulando l'espressione dei recettori per FSH e LH (Porter e al., 2000). Tuttavia i meccanismi attraverso i quali la prolattina esercita questi effetti, talvolta contrastanti, non sono ancora completamente conosciuti.

Nel nostro modello sperimentale abbiamo osservato un effetto inibitorio sulla produzione di entrambi gli steroidi, estradiolo 17 β e progesterone, quando le cellule della granulosa sono trattate con prolattina alle concentrazioni 1 e 10 nM.

Un effetto inibitorio diretto sull'attività steroidogenica basale era stato osservato anche da Cutie e Andino nel 1988 in cellule della granulosa umane.

La prolattina potrebbe esercitare un effetto simil-gonadotropinico e regolare la produzione degli steroidi nel follicolo antrale contribuendo alla fine regolazione della maturazione follicolare. Il tipo di effetto appare strettamente dipendente alla concentrazione raggiunta dalla proteina.

Occorre ricordare che, numerosi fattori intervengono nella regolazione dello sviluppo follicolare modulando un processo fondamentale per la crescita del follicolo, l'angiogenesi. Essi possono agire direttamente sulle cellule endoteliali oppure produrre effetti indiretti alterando la capacità della granulosa di produrre fattori che agiscono sui vasi follicolari regolandone lo sviluppo. Fra questi, un ruolo fondamentale è svolto dal VEGF. Diversi studi concordano nell'attribuire alla prolattina un ruolo nella regolazione del processo angiogenico (Clapp e al., 2008). Tuttavia, così come per la steroidogenesi, in letteratura, sono presenti dati discordanti. Infatti, alcuni studi hanno attribuito all'ormone un effetto proangiogenico (Gaytan e al., 1997; Ko e al., 2003; Hilfiker-kleiner e al., 2007), altri invece non evidenziano effetti sulla proliferazione delle cellule endoteliali (Struman e al., 1999; Ueda e al., 2006; Ricken e al., 2007).

I dati ottenuti in questo lavoro mostrano un effetto stimolatorio della proteina sulla crescita delle AOC in gel di fibrina. Un effetto positivo della prolattina sull' angiogenesi era stato dimostrato anche da

Malaguarnera e collaboratori (2002) nell'ambito di uno studio *in vitro* condotto su endoteliali umane.
In particolare, i risultati ottenuti in questo lavoro suggeriscono che la crescita delle AOC potrebbe essere mediata dalla capacità dell'ormone di aumentare la produzione di VEGF da parte delle cellule endoteliali stesse. Inoltre, abbiamo dimostrato che tale effetto viene prodotto anche sulle cellule della granulosa: la prolattina potrebbe quindi fungere da stimolatore fisiologico locale del processo di angiogenesi follicolare nel suino. Questa ipotesi è avvalorata da quanto recentemente evidenziato nella specie bovina da Castilla e collaboratori (2010).
In accordo con quanto osservato da Molinari e collaboratori (2007), i nostri dati evidenziano che la prolattina inibisce la produzione endoteliale di un altro importante mediatore angiogenico, l'ossido nitrico.
In realtà, il ruolo svolto da questa molecola nel controllo dell'angiogenesi è ancora controverso; in particolare, non è stata ancora chiarita l'azione che essa svolge sulla sintesi di VEGF. Infatti, alcuni autori riportano un'azione di tipo stimolatorio (Kimura e Esumi., 2003; Dulak e al., 2000) mentre altri ne dimostrano l'attività inibitoria (Powell e al., 2000). La concentrazione raggiunta dalla molecola appare determinante per il tipo di effetto. Infatti, livelli basali sembrano produrre l'attivazione del fattore di trascrizione HIF-1, e stimolare l'espressione genica del VEGF, mentre livelli elevati appaiono agire negativamente sulla sintesi di VEGF, probabilmente limitando l'azione di HIF-1 (Kimura e Esumi, 2003). Pertanto, la prolattina potrebbe mantenere la produzione di NO a un livello tale da favorire la produzione di VEGF.
Occorre evidenziare che mentre i meccanismi di controllo del rilascio ipofisario della prolattina sono stati individuati, le modalità di regolazione della sua produzione extraipofisaria non sono ancora noti.

Nel nostro lavoro abbiamo osservato come la produzione di prolattina da parte delle cellule della granulosa venga stimolata in modo significativo quando le cellule in coltura sono sottoposte ad una condizione ipossica: la prolattina prodotta a livello follicolare potrebbe pertanto essere coinvolta nella risposta cellulare all' ipossia e partecipare al controllo fisiologico del processo angiogenico stesso, un evento che appare fortemente stimolato dalla deprivazione di ossigeno, segnalata mediante eventi mitocondriali che si traducono in variazioni dei livelli delle specie reattive dell'ossigeno (ROS) (Basini e al., 2004).
È stato dimostrato che i ROS svolgono un ruolo importante nella regolazione dello sviluppo del follicolo ovarico (Basini e al, 2008). Tuttavia, l'accumulo e il raggiungimento di elevati livelli intracellulari di queste sostanze risulta dannoso per la funzionalità del follicolo e per il processo riproduttivo: esistono pertanto diversi sistemi deputati al mantenimento di livelli costanti di queste molecole. I dati che abbiamo ottenuto evidenziano che la prolattina è in grado di inibire la produzione dell' anione superossido e del perossido di idrogeno. L'effetto inibitorio sulla produzione dei ROS conferisce alla prolattina una potenziale funzione protettiva nei confronti dell'accumulo di queste sostanze nel follicolo. È stato evidenziato che la prolattina ha un effetto antiapoptotico in diversi tipi cellulari ed anche sulle cellule della granulosa umane per le quali funge da fattore di sopravvivenza (Perks e al., 2003): tale effetto potrebbe essere mediato dalla capacità di limitare la produzione di radicali liberi che nel periodo preovulatorio raggiunge livelli molto elevati. Ulteriori studi sono necessari per chiarire i meccanismi attraverso i quali la prolattina riduce la produzione dei ROS, dal momento che nel nostro lavoro non è stato dimostrato un parallelo incremento nell'attività degli enzimi deputati alla loro detossificazione: l'effetto inibitorio sulla produzione dei ROS, potrebbe

essere dovuto alla stimolazione dei sistemi di detossificazione non enzimatici.

In conclusione, i risultati ottenuti in questo studio dimostrano che la prolattina nel suino è in grado di modulare processi fondamentali per la funzionalità e lo sviluppo follicolare quali steroidogenesi ed angiogenesi. La presenza della proteina nel fluido follicolare e l'espressione del suo recettore nei follicoli piccoli sono indicativi di un ruolo regolativo fin dalle prime fasi dello sviluppo. Quando il follicolo raggiunge la crescita massima l'aumentato contenuto di prolattina nel fluido, appare possibile conseguenza della produzione locale stimolata dalle condizioni crescenti di ipossia che si realizzano durante lo sviluppo follicolare.

Funzionalità follicolare e angiogenesi: interferenti esogeni

Effetto del Gossipolo

Il Gossipolo è un polifenolo presente nella pianta del cotone del genere *Gossypium* della famiglia delle *Malvaceae* (Figura 22).

Figura 22 . Pianta del cotone.

La presenza di sei gruppi idrossilici fenolici e di due gruppi aldeidici rendono il composto chimicamente reattivo (Figura 23). La sua azione è stata associata a fenomeni di tossicità e, in varie specie, sono stati osservati effetti avversi sul sistema riproduttivo in seguito ad un eccessivo consumo (Santos e al., 2003; Fombad e al., 2004).

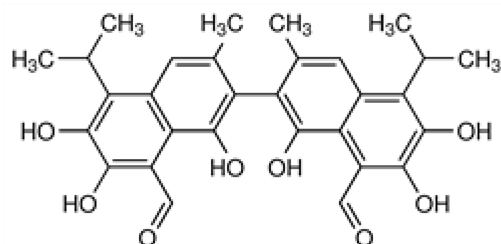


Figura 23. Struttura chimica del Gossipolo.

Tuttavia, i meccanismi molecolari attraverso i quali questo composto esercita la sua azione non sono ancora stati completamente chiariti. Inoltre, una maggiore conoscenza della sua attività biologica appare utile alla luce delle potenziali applicazioni terapeutiche di questa sostanza: sono state infatti attribuite al composto proprietà antitumorali, antimicrobiche, antivirali e antiossidanti (Wang e al., 2009). Nel lavoro "Gossipolo, a polyphenolic aldeide from cotton plant, interferes with swine granulosa cell function" (Allegato F, reperibile nella versione definitiva al link :
http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6T62-4VWPSX0—2&_user=606283&_coverDate=07%2F31%2F2009&_rdoc=1&_fmt=high&_orig=search&_origin=search&_sort=d&_docanchor=&view=c&_acct=C000031458&_version=1&_urlVersion=0&_userid=606283&md5=65652df8dc00ed1e7d70464567e58858&searchtype=a;
doi:10.1016/j.domaniend.2009.01.005) è stato condotto uno studio *in vitro* in cui sono stati indagati gli effetti del Gossipolo alle concentrazioni di 5 e 25 μg/ml sulle cellule della granulosa suine. Lo studio ha rivelato che il composto è in grado di modulare in modo significativo diversi parametri funzionali delle cellule follicolari: proliferazione, steroidogenesi, produzione di ossido nitrico, anione superossido e VEGF, l'attività dell'enzima superossido dismutasi e dei sistemi antiossidanti non enziamtici. In particolare, ha causato un inibizione significativa della steroidogenesi ($p < 0{,}001$). Tale effetto potrebbe essere mediato dall' aumento della produzione dei metaboliti dell'ossigeno, ossido nitrico e anione superossido ($p < 0{,}05$). A sua volta, l'effetto sulla produzione di anione superossido potrebbe essere dovuto all'inibizione dell'enzima deputato alla degradazione di questo radicale, la SOD ($p < 0{,}001$). Abbiamo inoltre dimostrato che la sostanza stimola la produzione del fattore angiogenico VEGF ($p < 0{,}001$) e l'attività dei sistemi di detossificazione non enzimatici dei radicali liberi ($p < 0{,}001$).

Dall’analisi complessiva dei risultati ottenuti è emerso che il Gossipolo è in grado di alterare l’attività delle cellule della granulosa *in vitro* e pertanto potrebbe fungere da interferente nella regolazione della funzionalità ovarica nel suino.

Effetto del Bisfenolo A

Il bisfenolo A (BPA) è uno degli EDC maggiormente studiati. Esso appartiene al gruppo degli xenoestrogeni poiché mima l'azione degli estrogeni interagendo con i loro recettori nucleari ER α e β. Il BPA è una sostanza di sintesi (Figura 24) utilizzata prevalentemente per la produzione di un tipo di plastica impiegata nella realizzazione di recipienti per alimenti e bevande. In seguito a fenomeni di esposizione alla luce, invecchiamento, riscaldamento e contatto con composti acidi o basici questa sostanza può essere rilasciata nell'ambiente. La sua presenza è stata riscontrata in diversi campioni biologici fra cui saliva, siero, fluido follicolare e urine (Ikezuki e al., 2002; Calafat e al., 2008).

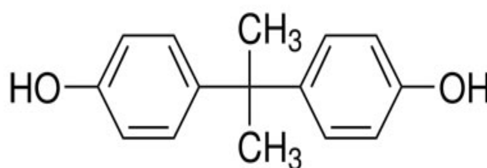


Figura 24. Struttura chimica del Bisfenolo A.

Gli studi sull'attività biologica del composto hanno evidenziato alterazioni a carico del sistema riproduttivo causate dall'esposizione a questa sostanza (Maffini e al., 2006). È stata inoltre dimostrata una sua potenziale interazione con il processo angiogenico (Long e al., 2001). Nel lavoro "Bisphenol A disrupts granulosa cell function" (Allegato G; reperibile nella versione definitiva al link: http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6T62-4YC7G17—1&_user=606283&_coverDate=07%2F31%2F2010&_rdoc=1&_fmt=high&_orig=search&_origin=search&_sort=d&_docanchor=&view=c&_acct=C000031458&_version=1&_urlVersion=0&_userid=606283&

md5=22db42514c65e663b7f59f4374c37bb4&searchtype=a; doi:10.1016/j.domaniend.2010.01.004) è stato effettuato uno studio *in vitro* allo scopo di indagare la capacità del BPA di interferire nella regolazione fisiologica dello sviluppo del follicolo antrale suino e del processo di angiogenesi ovarica. In particolare, è stato valutato l'effetto di tre diverse concentrazioni di BPA (0,1 - 1 e 10 μM) su parametri funzionali delle cellule della granulosa quali proliferazione, produzione di steroidi, produzione di VEGF e stato redox. Dall'analisi dei risultati è emerso che il BPA non ha effetto su proliferazione e sullo stato redox della granulosa. Al contrario, la produzione di E2 è stata stimolata ($p<0,001$) in presenza di BPA 0,1 μM, mentre in presenza delle concentrazioni più alte si è verificato l'effetto opposto ($p<0,001$). Il BPA ha inoltre causato un inibizione significativa ($p<0,01$) della produzione di P4 a tutte le concentrazioni testate ed un effetto promotore sulla produzione di VEGF ($p<0,05$). In base ai risultati ottenuti è possibile concludere che il BPA è un composto potenzialmente in grado di alterare la funzionalità delle cellule della granulosa e, considerato il ruolo fondamentale di queste cellule nell'attività ovarica, l'efficienza riproduttiva. Il BPA potrebbe inoltre essere promotore di uno sviluppo vascolare incontrollato.

Il follicolo ovarico: un modello per lo studio di potenziali agenti terapeutici

Quando i meccanismi di controllo dell' angiogenesi fisiologica vengono meno si assiste ad uno sviluppo vascolare incontrollato che caratterizza l'insorgenza di diverse patologie. Per la terapia delle malattie caratterizzate da uno sviluppo vascolare eccessivo quali cancro, retinopatie e artrite, è necessario utilizzare degli agenti antiangiogenici che consentano di bloccare la crescita dei vasi sanguigni. Pertanto, nell' ultimo decennio sono stati fatti numerosi sforzi nel tentativo di individuare composti dotati di questa proprietà. Sono stati sviluppati diversi inibitori angiogenici la cui efficacia viene valutata in un primo momento mediante l'esecuzione di test *in vitro* e *in vivo* e successivamente attraverso una valutazione clinica. Recenti studi hanno evidenziato che numerosi componenti bioattivi presenti nelle piante e negli alimenti rappresentano una potenziale fonte di agenti antiangiogenici (Varinska e al., 2010). Essi inibiscono l'angiogenesi attraverso diversi meccanismi: modulano la produzione delle citochine e dei fattori di crescita proangiogenci, modulano parametri funzionali dell'endotelio (proliferazione e migrazione) e i segnali di comunicazione intracellulari ed extracellulari (Davis e al., 2010). Tuttavia, perché le molecole bioattive isolate raggiungano un' efficacia terapeutica, spesso, è necessario apportare delle modifiche chimiche al composto naturale. Dopo tali cambiamenti strutturali è necessario verificare che l'effetto desiderato venga mantenuto dal derivato sintetico.

L'E-resveratrolo è un polifenolo appartenente al gruppo degli stilbenoidi presente ad elevate concentrazioni nel vino rosso. Gli studi effettuati hanno attribuito al composto un ruolo potenziale nella prevenzione delle malattie cardiovascolari e del cancro. Esso infatti è dotato di proprietà antiproliferative (Benitez e al., 2007), antiangiogeniche (Zhang e al., 2005) ed antiinfiammatorie (Nassiri-Asl e Hosseinzadeh,, 2009). Poiché il composto ha una bassa biodisponibilità, nel tentativo di migliorare la

sua attività e la potenza del suo effetto terapeutico sono stati sviluppati diversi analoghi, fra questi i polimetossistilbeni rappresentano il sottogruppo principale. Nel lavoro "Antiangiogenic Resveratrol Analogues by Mild m-CPBA Aromatic Hydroxylation of 3,5-Dimethoxystilbenes" (Allegato H, reperibile nella versione definitiva al link: http://www.ncbi.nlm.nih.gov/pubmed/19370931) è stato condotto uno studio *in vitro* per verificare le proprietà antiangiogeniche di quattro analoghi dell' E-resveratrolo:

- 3,5,4'-trimetossistilbene (2),
- 2-idrossi-3,5,4'-trimetossistilbene (5),
- 2-idrossi-3,5,3',5'-tetrametossistilbene (8)
- 2-idrossi-3,5,3',4'-tetrametossistilbene (11).

In particolare, per ogni composto, (concentrazioni testate 0,1 – 1 – 10 e 100 μM) è stato valutato l'effetto sulla crescita delle AOC in gel di fibrina. I composti 2, 5 e 8 hanno mostrato un effetto inibitorio significativo ($p<0,001$) sulla crescita endoteliale a tutte la concentrazioni testate; il composto 11, alla concentrazione più bassa, ha invece avuto un effetto inibitorio significativo solo dopo 96h. I risultati ottenuti dimostrano che gli analoghi di sintesi del resveratrolo posseggono effetto antiangiogenico; poiché le modificazioni chimiche conferiscono a questi composti una maggiore biodisponibilità rispetto al resveratrolo essi potrebbero fungere da potenziali agenti antiangiogenici utilizzabili per la terapia *in vivo*. In uno studio successivo "Biological effects on granulosa cells of hydroxylated and metilated resveratrol analogues" (Allegato I, reperibile nella versione definitiva al link:
http://onlinelibrary.wiley.com/doi/10.1002/mnfr.200900320/pdf;
doi:10.1002/mnfr.200900320) sono stati selezionati due dei composti che nello studio precedente avevano mostrato una potente attività

antiangiogenica: 3,5,4'- trimetossistilbene (2) e 2-idrossi-3,5,4'-trimetossistilbene (3). Essi sono stati impiegati in uno studio *in vitro* allo scopo di determinare eventuali effetti biologici su processi che caratterizzano l'attività funzionale delle cellule della granulosa suine fra cui l'angiogenesi follicolare. In particolare, sono stati valutati gli effetti (concentrazioni testate 0,1- 1-10- e 100 μM) su vitalità, produzione di steroidi, produzione di VEGF e stato redox . Lo studio ha rilevato che il composto 3 è in grado di inibire la crescita delle cellule della granulosa e di stimolare la produzione degli steroidi. Lo stesso effetto sulla proliferazione e sulla produzione di estradiolo 17-β è stato osservato in presenza del composto 2 alla concentrazione più alta, mentre lo stesso dosaggio ha inibito in modo significativo la produzione di progesterone. Entrambi gli analoghi hanno inibito la produzione di VEGF. Infine lo stato redox non è stato modulato in modo significativo dal composto 2 mentre il composto 3, alla concentrazione 100 μM, ha stimolato la generazione di radicali liberi e l'attività degli enzimi di detossificazione. I dati ottenuti incrementano le conoscenze relative agli effetti biologici dei polimetossistilbeni e assumono interesse in relazione al loro potenziale utilizzo a scopo terapeutico.

Il CAPE (estere feniletilico dell'acido caffeico) è un componente bioattivo della propoli, sostanza resinosa prodotta dalle api. Esso è dotato di proprietà antiossidanti, antiproliferative (Jaganathan e Mandal, 2009) e antiangiogeniche (Ahn e al., 2009). Nello studio "Antiangiogenic properties of an unusual benzo [k.l] xantene lignan derived from CAPE (Caffeic acid Phenethyl Ester)" (Allegato L; http://www.springerlink.com/content/084x161561705832/fulltext.pdf ; doi:10.1007/s10637-010-9550-z) attraverso una dimerizzazione biomimetica del CAPE è stato ottenuto un nuovo derivato sintetico, il benzo [k.l] xantene, un lignano la cui struttura è correlata a un raro

gruppo di composti naturali. Per verificare il mantenimento delle proprietà antiangiogeniche del composto è stato effettuato uno studio *in vitro.* In particolare, sono stati valutati gli effetti dell'analogo sintetico (concentrazioni testate 0,1-1 e 10 μM) sulla crescita delle AOC nel saggio di angiogenesi in gel di fibrina e sulla produzione del VEGF da parte delle cellule della granulosa suine. Il composto ha mostrato un significativo effetto inibitorio ($p<0,05$), dose dipendente, sulla crescita delle cellule endoteliali. Un effetto inibitorio significativo è stato osservato anche sulla produzione di VEGF alla concentrazione 10 μM ($p<0,001$). Questi dati motivano ad uno studio più approfondito al fine di qualificare il benzo [k.l] xantene come nuovo farmaco antiangiogenico.

BIBLIOGRAFIA

Acosta T.J., Hayashi K.G., Ohtani M., Miyamoto A. Local changes in blood flow within the preovulatory follicle wall and early corpus luteum in cows. 2003. Reproduction. 125: 759–767.

Adams R.H., Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. 2007. Nat. Rev. Mol. Cell Biol. 8: 464–478.

Agarwal A., Gupta S., Sharma R.K. Role of oxidative stress in female reproduction. 2005. Reprod. Biol. Endocrinol. 14: 3-28.

Ahn M.R., Kunimasa K., Kumazawa S., Nakayama T., Kaji K., Uto Y., Hori H., Nagasawa H., Ohta T. Correlation between antiangiogenic activity and antioxidant activity of various components from propolis. 2009. Mol. Nutr. Food Res. 53: 643-651.

Albini A., Brigati C., Ventura A., Lorusso G., Pinter M., Morini M., Mancino A., Sica A., Noonan D.M. Angiostatin anti-angiogenesis requires IL-12: the innate immune system as a key target. 2009. J. Transl. Med. 7: 5.

Ali S., Pellegrini I., Kelly P.A. A prolactin-dependent immune cell line (Nb2) expresses a mutant form of prolactin receptor. 1991. J. Biol. Chem. 266: 20110–20117.

Allende M.L., Yamashita T., Proia R.L. G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation. 2003. Blood. 102: 3665–3667.

Armulik A., Abramsson A., Betsholtz C. Endothelial/pericyte interactions. 2005. Circ. Res. 97: 512–523.

Barboni B., Turriani M., Galeati G., Spinaci M, Bacci M.L., Forni M., Mattioli M. Vascular endothelial growth factor production in growing pig antral follicles. 2000. Biol. Reprod. 63: 858-864.

Basini G., Bianco F., Grasselli F., Tirelli M., Bussolati S., Tamanini C. The effects of reduced oxygen tension on swine granulosa cell. 2004. Regul. Pept. 120: 69-75.

Basini G., Santini S.E, Grasselli F. 2- methoxyestradiol inhibits superoxide anion generation while it enhances superoxide dismutase activity in swine granulosa cells. 2006. Ann. N. Y. Acad. Sci. 1091: 34-40.

Basini G., Bussolati S., Santini S.E., Bianchi F., Careri M., Mangia A., Musci M., Grasselli F. Antiangiogenesis in swine ovarian follicle: a potential role for 2-methoxyestradiol. 2007. Steroids. 72: 660-665.

Basini G., Bussolati S., Santini S.E., Bianchi F., Careri M., Mangia A., Musci M., Grasselli F. Hydroxyestrogens inhibit angiogenesis in swine ovarian follicles. 2008a. J. Endocrinol. 199:127-135.

Basini G., Simona B., Santini S.E., Grasselli F. Reactive oxygen species and anti-oxidant defences in swine follicular fluids. 2008b. Reprod. Fertil. Dev. 20:269-274

Basini G., Bussolati S., Santini S.E., Grasselli F. Stanniocalcin, a potential ovarian angiogenesis regulator, does not affect endothelial cell apoptosis. 2009. Ann. N. Y. Acad. Sci. 1171: 94-99.

Benitez D.A., Pozo-Guisado E., Alvarez-Barrientos A., Fernandez-Salguero P.M., Castellón E.A. Mechanisms involved in resveratrol-induced apoptosis and cell cycle arrest in prostate cancer-derived cell lines. 2007. J. Androl. 28: 282-293.

Benzie I.F., Strain J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. 1996. Anal. Biochem. 239: 70-76.

Berisha B., Steffl M., Welter H., Kliem H., Meyer H.H., Schams D., Amselgruber W. Effect of the luteinising hormone surge onregulation of vascular endothelial growth factor and extracellularmatrix-degrading proteinases and their inhibitors in bovine follicles. 2008. Reprod. Fertil. Dev. 20: 258–268.

Bertolino P., Deckers M., Lebrin F., ten Dijke P. Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders. 2005. Chest. 128: 585S–590S.

Bianco F., Basini G., Santini S., Grasselli F. Angiogenic activity of swine granulosa cells: effects of hypoxia and the role of VEGF. 2005. Vet. Res. Commun. 2:157-159.

Binart N., Bachelot A., Bouilly J. Impact of prolactin receptor isoforms on reproduction. 2010. Trends. Endocrinol. Metab. 21: 362-368.

Bole-Feysot C., Goffin V., Edery M., Binart N., Kelly P. Prolactin (prl) and its receptor: actions, signal transduction pathways and phenotypes observed in prl receptor knockout mice. 1998. Endocr. Rev. 19: 225–268.

Cadenas E. Basic mechanisms of antioxidant activity. 1997. Biofactors. 6: 391–397.

Calafat A.M., Weuve J., Ye X., Jia L.T., Hu H., Ringer S., Huttner K., Hauser R. Exposure to bisphenol A and other phenols in neonatal intensive care unit premature infants. 2009. Environ. Health Perspect. 117: 639-644.

Carmeliet P., Jain R.K. Angiogenesis in cancer and other diseases. 2000. Nature. 407: 249-257.

Castilla A., García C., Cruz-Soto M., Martínez de la Escalera G., Thebault S., Clapp C. Prolactin in ovarian follicular fluid stimulates endothelial cell proliferation. 2010. J. Vasc. Res. 47: 45-53.

Chakraborty A., Elbjeirami W., Sheikh-Hamad D., McReynolds M.R., Hoying J.B., Bick R., Truong L., Poindexter B., Hui L., Brooks H., Zhang P., Smith W. Stanniocalcin-1 regulates endothelial gene expression and modulates transendothelial migration of leukocytes. 2007. Am. J. Physiol. Renal. Physiol. 292: 895-904.

Chang A.C., Janosi J., Hulsbeek M., de Jong D., Jefrey K.J, Noble J.R., Reddel R.R. A novel human cDNA highly homologous to the fish hormone stanniocalcin. 1995. Mol. Cell. Endocrinol. 112: 241-247.

Clapp C., Thebault S., Martínez de la Escalera G. Role of prolactin and vasoinhibins in the regulation of vascular function in mammary gland. 2008. J. Mammary Gland Biol. Neoplasia. 13: 55-67.

Clapp C., Thebault S., Jeziorski M. C., De la Escalera G.M. Peptide Hormone Regulation of Angiogenesis. 2009. Physiol. Rev. 89: 1177–1215,

Cooke N.E., Coit D., Shine J., Baxter J.D., Martial J.A. Human prolactin cDNA structural analysis and evolutionary comparisons. 1981. J. Biol. Chem. 256: 4007–4016.

Corbacho A.M., Macotela Y., Nava G., Torner L., Dueñas Z., Noris G., Morales M.A., Martínez De La Escalera G., Clapp C. Human umbilical vein endothelial cells express multiple prolactin isoforms. 2000. J. Endocrinol. 166: 53-62.

Costa C., Soares R., Schmitt F.. Angiogenesis: now and then. 2004. Apmis. 112: 402-412.

Cutie E., Andino N.A. Prolactin inhibits the steroidogenesis in midfollicular phase human granulosa cells cultured in a chemically defined medium. 1988. Fertil. Steril. 49: 632-637.

Davis C.D., Emenaker N.J., Milner J.A. Cellular proliferation, apoptosis and angiogenesis: molecular targets for nutritional preemption of cancer. Semin. Oncol. 2010. 37: 243-257.

Davis J.A., Linzer D.I.H. Expression of multiple forms of the prolactin receptor in mouse liver. 1989. Mol. Endocrinol. 3: 674–680.

Deol H.K., Varghese R., Wagner G.F., Dimattia G.E. Dynamic regulation of mouse ovarian stanniocalcin expression during gestation and lactation. 2000. Endocrinology. 141: 3412-3421.

Dimmeler S., Zeiher A.M. Endothelial cell apoptosis in angiogenesis and vessel regression. 2000. Circ. Res. 87: 434-439.

Distler J.H., Hirth A., Kurowska-Stolarska M., Gay R.E., Gay S., Distler O. Angiogenic and angiostatic factors in the molecular control of angiogenesis. 2003. Q. J. Nucl. Med. 47:149-161.

Drummond A.E. The role of steroids in follicular growth. 2006. Reprod. Biol. Endocrinol. 4: 16.

Dulak J., Józkowicz A., Dembinska-Kiec A., Guevara I., Zdzienicka A., Zmudzinska-Grochot D., Florek I., Wójtowicz A., Szuba A., Cooke

J.P. Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. 2000. Arterioscler. Thromb. Vasc. Biol. 20: 659-666.

Duncan W.C., Van den Driesche S., Fraser H.M. Inhibition of vascular endothelial growth factor in the primate ovary up-regulates hypoxia inducible factor-1 alpha in the follicle and corpus luteum. 2008. Endocrinology.149: 3313–3320.

Einspanier R., Pitzel L., Wuttke W., Hagendorff G., Preuss K.D., Kardalinou E., Scheit K.H. Demonstration of mRNAs for oxytocin and prolactin in porcine granulosa and luteal cells. Effects of these hormones on progesterone secretion in vitro. 1986. FEBS Lett. 204: 37-40.

Engelkamp D. Cloning of three mouse Unc5 genes and their expression patterns at mid-gestation. 2002. Mech. Dev. 118: 191–197.

Engelse M.A., Laurens N., Verloop R.E., Koolwijk P., van Hinsbergh V.W. Differential gene expression analysis of tubule forming and non-tubule forming endothelial cells: CDC42GAP as a counter-regulator in tubule formation. 2008. Angiogenesis. 11:153–167.

Erdmann S., Ricken A., Merkwitz C., Struman I., Castino R., Hummitzsch K., Gaunitz F., Isidoro C., Martial J., Spanel-Borowski K. The expression of prolactin and its cathepsin D-mediated cleavage in the bovine corpus luteum vary with the estrous cycle. 2007. Am. J. Physiol. Endocrinol. Metab. 293: E1365-1377.

Farmer C., Palin M.F. Exogenous prolactin stimulates mammary development and alters expression of prolactin-related genes in prepubertal gilts. 2005. J. Anim. Sci. 83: 825-832.

Findlay J.K., Kerr J.B., Britt K., Liew S.H., Simpson E.R., Rosairo D., Drummond A. Ovarian physiology: follicle development, oocyte and hormone relationships. 2009. Anim. Reprod. 6: 16-19.

Fombad R.B., Bryant M.J. An evaluation of the use of cottonseed cake in the diet of growing pigs. 2004. Trop. Anim. Health Prod. 36: 295-305.

Francis M.E, Uriel S., Brey E.M. Endothelial cell-matrix interactions in neovascularization. 2008. Tissue Eng Part B Rev. 14:19–32.

Fraser H.M. Regulation of the ovarian follicular vasculature. 2006. Reprod. Biol. Endocrinol. 12: 4-18.

Fraser H.M., Duncan W.C. SRB Reproduction, Fertility and Development Award Lecture 2008. Regulation and manipulation of angiogenesis in the ovary and endometrium. 2009. Reprod. Fertil. Dev. 21: 377-392.

Freeman M.E., La Kanyicska B., Lerant A., Nagy G. Prolactin: structure, function, and regulation of secretion. 2000. Physiol. Rev. 80: 1523-1631.

Gaytan F., Morales C., Bellido C., Aguilar E., Sanchez-Criado J.E. Role of prolactin in the regulation of macrophages and in the proliferative activity of vascular cells in newly formed and regressing rat corpora lutea. 1997. Biol. Reprod. 57: 478-486.

Gerritsen M.E., Soriano R., Yang S., Ingle G., Zlot C., Toy K., Winer J., Draksharapu A., Peale F., Wu T.D., Williams P.M. In silico data filtering to identify new angiogenesis targets from a large in vitro gene profiling data set. 2002. Physiol. Genomics. 9: 2-25.

Gerritsen M.E., Wagner G.F. Stanniocalcin: no just a fish tale. 2005. Vitam. Hormon. 70: 105-135.

Geva E., Jaffe R.B. 2004. In: The ovary. 2nd ed. Adashi E., Leung P.C.K (ed.). Elsevier Academic Press, S. Diego, USA. pp 305-314.

Gonzalez C., Parra A., Ramírez-Peredo J., García C., Rivera C.J.´, Macotela Y., Aranda J., Lemini M., Arias J., Ibargüengoitia F., Martínez de la Escalera G., Clapp C. Elevated vasoinhibins may contribute to endothelial cell dysfunction and low birth weight in preeclampsia. 2007. Lab. Invest. 87: 1009–1017.

Grasselli F., Baratta M., Tamanini C. Effects of GnRH analogue (buserelin) infused via osmotic minipumps on pituary and ovarian activity of prepuberal heifers. 1993. Anim. Reprod. Sci. 32: 153-161.

Grasselli F., Basini G., Tirelli M., Cavalli V., Bussolati S., Tamanini C. Angiogenic activity of porcine granulosa cells co-cultured with endothelial cells in a microcarrier-based three-dimensional fibrin gel. 2003. J. Physiol. Pharmacol. 54: 361-370.

Grattan D.R., Kokay I.C. Prolactin: A Pleiotropic Neuroendocrine Hormone. 2008. J. Neuroendocrinol. 20: 752–763.

Greenaway J., Gentry P.A., Feige J.J., LaMarre J., Petrik J.J. Thrombospondin and vascular endothelial growth factor are cyclically expressed in an inverse pattern during bovine ovarian follicle development. 2005. Biol. Reprod. 72: 1071–1078.

Greenberg J.I., Shields D.J., Barillas S.G., Acevedo L.M., Murphy E., Huang J.H., Scheppke L., Stockmann C., Johnson R.S., Angle N. et al. A role for VEGF as a negative regulator of pericyte function and vessel maturation. 2008. Nature. 456: 809–813.

Gridley T. Notch signaling in vascular development and physiology. 2007. Development. 134: 2709–2718.

Groothuis P.G. Angiogenesis and vascular remodelling in female reproductive organs. 2005. Angiogenesis. 8: 87–88.

Havelock J.C., Rainey W.E., Carr B.R. Ovarian granulosa cell lines. 2004. Mol. Cell. Endocrinol. 228: 67–78.

Hayashi K.G., Berisha B., Matsui M., Schams D., Miyamoto A. Expression of mRNA for the angiopoietin-Tie system in granulosa cells during follicular development in cows. 2004. J. Reprod. Dev. 50: 477–480.

Hellstrom M., Phng L.K., Hofmann J.J. Wallgard E., Coultas L., Lindblom P., Alva J., Nilsson A.K., Karlsson L., Gaiano N., Yoon K., Rossant J., Iruela-Arispe M.L., Kalén M., Gerhardt H., Betsholtz C. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. 2007. Nature. 445: 776–780.

Henderson K.M., McNeilly A.S., Swanston I.A. Gonadotrophin and steroid concentrations in bovine follicular fluid and their relationship to follicle size. 1982. J. Reprod. Fertil. 65: 467-73.

Hilfiker-Kleiner D., Kaminski K., Podewski E., Bonda T., Schaefer A., Sliwa K., Forster O., Quint A., Landmesser U., Doerries C., Luchtefeld M., Poli V., Schneider M.D., Balligand J.L., Desjardins F., Ansari A., Struman I., Nguyen N.Q., Zschemisch N.H., Klein G., Heusch G., Schulz R., Hilfiker A., Drexler H. A cathepsin D-cleaved 16 kDa form of prolactin mediates postpartum cardiomyopathy. 2007. Cell. 128: 589-600.

Ikezuki Y., Tsutsumi O., Takai Y., Kamei Y., Taketani Y. Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. 2002. Hum. Reprod. 17: 2839-2841.

Ishibashi K., Imai M. Prospect of a stanniocalcin endocrine/paracrine system in mammals. 2000. Am. J. Physiol. Renal. Physiol. 282: F367-F375.

Jaganathan S.K., Mandal M. Antiproliferative effects of honey and of its polyphenols: a review. 2009. J. Biomed. Biotechnol. 2009: 830616.

Jaggers D.C., Collins W.P., Milligan S.R. Potent inhibitory effects of steroids in an in vitro model of angiogenesis. 1996. J. Endocrinol. 150: 457-464.

Jamnongjit M., Gill A., Hammes S.R. Epidermal Growth Factor Receptor signaling is required for normal ovarian steroidogenesis and oocyte maturation. 2005. Proc. Natl. Acad. Sci. 102: 16257-16261.

Jamnongjit M., Hammes S.R. Ovarian Steroids: The good, the bad, and the signals that raise them. 2006. Cell Cycle. 5: 1178–1183.

Jiang J.Y., Macchiarelli G., Tsang B.K., Sato E. Capillary angiogenesis and degeneration in bovine ovarian antral follicles. 2003. Reproduction. 125: 211–223.

Kamei M, Saunders W.B., Bayless K.J., Dye L., Davis G.E., Weinstein B.M. Endothelial tubes assemble from intracellular vacuoles in vivo. 2006. Nature. 442: 453–456.

Kappler J., Franken S., Junghans U., Hoffmann R., Linke T., Müller H.W., Koch K.W. Glycosaminoglycan-binding properties and secondary structure of the C-terminus of netrin-1. 2000. Biochem. Biophys. Res. Commun. 271: 287-291.

Kelly P.A., Djiane J., Postel-Vinay M.C., Edery M. The prolactin/ growth hormone receptor family. 1991. Endocr. Rev. 12: 235–251.

Kennedy T.E., Serafini T., de la Torre J.R., Tessier-Lavigne M. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. 1994. Cell. 78: 425-435.

Kim J., Bagchi I., Bagchi M.. Signaling by hypoxia-inducible factors is critical for ovulation in mice. 2009. Endocrinology. 50: 3392–3400.

Kim K.H., Moriarty K., Bender J.R. Vascular cell signaling by membrane estrogen receptors. 2008. Steroids. 73: 864–869.

Kimura H., Esumi H. Reciprocal regulation between nitric oxide and vascular endothelial growth factor in angiogenesis. 2003. Acta Biochim. Pol. 50: 49-59.

Ko J.Y., Ahn Y.L., Cho B.N. Angiogenesis and white blood cell proliferation induced in mice by injection of a prolactin-expressing plasmid into muscle. 2003. Mol. Cells. 15: 262-270.

Koch M. , Murrel J.R., Hunter D.D., Olson P.F., Jin W., Keene D.R., Brunken W.J, Burgeson R.E. A Novel Member of the Netrin Family, ß-Netrin, Shares Homology with the ß Chain of Laminin: Identification, Expression, and Functional Characterization. 2000. J. Cell Biol. 151: 221-234.

Lamalice L., Le Boeuf F., Huot J. Endothelial cell migration during angiogenesis. 2007. Circ. Res. 100: 782–794.

Lehoux S., Castier Y., Tedgui A. Molecular mechanisms of the vascular responses to haemodynamic forces. 2006. J. Intern. Med. 259: 381–392.

Lesueur L., Edery M., Ali S., Paly J., Kelly P.A., Djiane J. Comparison of long and short forms of the prolactin receptor on

prolactin-induced milk protein gene transcription. 1991. Proc. Natl. Acad. Sci. USA. 88: 824–828.

Liu D., Jia H., Holmes D.I., Stannard A., Zachary I. Vascular endothelial growth factor-regulated gene expression in endothelial cells: KDR-mediated induction of Egr3 and the related nuclear receptors Nur77, Nurr1, and Nor1. 2003. Arterioscler. Thromb. Vasc. Biol. 23: 2002-2007.

Liu T.Z., Chin N., Kiser M.D., Bigler W.N. Specific spectrophotometry of ascorbic acid in serum or plasma by use of ascorbate oxidase. 1982. Clin. Chem. 28: 2225-2228.

Long X., Burke K.A., Bigsby R.M., Nephew K.P. Effects of the xenoestrogen bisphenol A on expression of vascular endothelial growth factor (VEGF) in the rat. 2001. Exp. Biol. Med. (Maywood). 226: 477-483.

Lu X., Le Noble F., Yuan L., Jiang Q., De Lafarge B., Sugiyama D., Bréant C., Claes F., De Smet F., Thomas J.L., Autiero M., Carmeliet P., Tessier-Lavigne M., Eichmann A. The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. 2004. Nature. 432: 179-186.

Maeda A., Matsuda F., Goto Y., Cheng Y., Gonda H., Inoue N., Nakagawa S. and Manabe N. Molecular Cloning of a Porcine (Sus scrofa) Apoptosis Inhibitory Ligand, Netrin-1, and Its Receptor, p53RDL1. 2008. J. Reprod. Dev. 54: 275-280.

Maffini M.V., Rubin B.S., Sonnenschein C., Soto A.M. Endocrine disruptors and reproductive health: the case of bisphenol-A. 2006. Mol. Cell Endocrinol. 254-255:179-186.

Malaguarnera L., Pilastro M.R., Quan S., Ghattas M.H., Yang L., Mezentsev A.V., Kushida T., Abraham N.G., Kappas A. Significance of heme oxygenase in prolactin-mediated cell proliferation and angiogenesis in human endothelial cells. 2002. Int. J. Mol. Med. 10: 433-440.

Martelli A., Berardinelli P., Russo V., Mauro A., Bernabo N., Gioia L., Mattioli M., Barboni B. Spatio-temporal analysis of vascular endothelial growth factor expression and blood vessel remodelling in pig ovarian follicles during the periovulatory period. 2006. J. Mol. Endocrinol. 36: 107–119.

Martelli A., Palmerini M.G., Russo V., Rinaldi C., Bernabo` N., Di Giacinto O., Berardinelli P., Nottola S.A., Macchiarelli G., Barboni B. Vessel remodelling in pig ovarian follicles during the periovulatory period: an immunohistochemistry and SEM-corrosion casting study. 2009. Reprod. Biol.and Endocrinol.. 7:72.

Miyashita T., Nishimura-Akiyoshi S., Itohara S., Rockland K. S. Strong expression of NETRIN-G2 in the monkey claustrum. 2005. Neurosc. 136: 487–496.

Molinari C., Grossini E., Mary D.A., Uberti F., Ghigo E., Ribichini F., Surico N., Vacca G. Prolactin induces regional vasoconstriction through the beta2-adrenergic and nitric oxide mechanisms. 2007. Endocrinology. 148: 4080-4090.

Nassiri-Asl M., Hosseinzadeh H. Review of the pharmacological effects of Vitis vinifera (Grape) and its bioactive compounds. 2009. Phytother. Res. 23: 1197-1204.

Neeman N., Abramovitch R., Schiffenbauer Y.S., Tempel C. Regulation of angiogenesis by hypoxic stress: from solid tumors to the ovarian follicle. 1997. Int. J. Exp. Path. 78: 57-70.

Niu P., Radman D., Jaworski E., Deol H., Gentz R.J.S., Olsen H., Wagner G.F. Development of a human stanniocalcin radioimmunoassay: serum and tissue hormone levels and pharmacokinetics in the rat. 2000. Mol. Cell. Endocrinol. 162: 131-144.

Nyberg P., Xie L., Kalluri R. Endogenous inhibitors of angiogenesis. 2005. Cancer Res. 65: 3967–3979.

Ochoa A., Montes de Oca P., Rivera J.C., Dueñas Z., Nava G., de La Escalera G.M., Clapp C. Expression of prolactin gene and secretion

of prolactin by rat retinal capillary endothelial cells. 2001. Invest. Ophthalmol. Vis. Sci. 42: 1639-1645.

Olsen H.S., Cepeda M.A., Zhang Q.Q., Rosen C.A., Vozzolo B.L. Human stanniocalcin: a possible hormonal regulator of mineral metabolism. 1996. Proc. Natl. Acad. Sci. 93: 1792-1796.

Park K.W., Crouse D., Lee M., Karnik S.K., Sorensen L.K., Murphy K.J., Kuo C.J., Li D.Y. The axonal attractant Netrin-1 is an angiogenic factor. 2004. Proc. Natl. Acad. Sci U S A. 101: 16210-16215.

Perks C.M., Newcomb P.V., Grohmann M., Wright R.J., Mason H.D., Holly J.M. Prolactin acts as a potent survival factor against C2-ceramide-induced apoptosis in human granulosa cells. 2003. Hum. Reprod. 18: 2672-2677.

Phelps J.Y., Bugg E.M., Shamblott M.J., Vlahos N.P., Whelan J., Zacur H.A. Prolactin gene expression in human ovarian follicular cells. 2003. Fertil. Steril. 79: 182-185.

Porter M.B., Brumsted J.R., Sites C.K. Effect of prolactin on follicle-stimulating hormone receptor binding and progesterone production in cultured porcine granulosa cells. 2000. Fertil. Steril. 73: 99-105.

Powell J.A., Mohamed S.N., Kerr J.S., Mousa S.A. Antiangiogenesis efficacy of nitric oxide donors. 2000. J. Cell Biochem. 80: 104-114.

Pugh C.W., Ratcliffe P.J. Regulation of angiogenesis by hypoxia: role of the HIF system. 2003. Nat. Med. 9: 677–684.

Reisinger K., Baal N, McKinnon T, Munstedt K, Zygmunt M. The gonadotropins: tissue-specific angiogenic factors? 2007. Mol. Cell. Endocrinol. 269: 65–80.

Ricken A.M., Traenkner A., Merkwitz C., Hummitzsch K., Grosche J., Spanel-Borowski K. The short prolactin receptor predominates in endothelial cells of micro- and macrovascular origin. 2007. J. Vasc. Res. 44: 19-30.

Riha G.M., Lin P.H., Lumsden A.B., Yao Q., Chen C. Roles of hemodynamic forces in vascular cell differentiation. 2005. Ann Biomed Eng. 33: 772–779.

Risau W. Mechanisms of angiogenesis. 1997. Nature. 386: 671–674.

Robinson R.S., Nicklin L.T., Hammond A.J., Schams D., Hunter M.G., Mann G.E. Fibroblast growth factor 2 is more dynamic than vascular endothelial growth factor A during the follicle–luteal transition in the cow. 2007. Biol. Reprod. 77: 28–36.

Robinson R.S., Woad K.J, Hammond A.J, Laird M., Hunter M.G, Mann G.E. Angiogenesis and vascular function in the ovary. 2009. Reproduction. 138: 869–881.

Sandau K.B., Fandrey J., Brüne B. Accumulation of HIF-1alpha under the influence of nitric oxide. 2001. Blood. 97:1009-1015.

Santos J.E., Villasenor M., Robinson P.H., DePeters E.J., Holmberg C.A. Type of cottonseed and level of gossypol in diets of lactating dairy cows: plasma gossypol, health, and reproductive performance. 2003. J. Dairy Sci. 86: 892-905.

Schams D., Berisha B. Steroids as local regulators of ovarian activity in domestic animals. 2002. Dom. Anim. Endocrinol. 23: 53–65.

Serafini T., Colamarino S.A., Leonardo E.D., Wang H., Beddington R., Skarnes W.C., Tessier-Lavigne M. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. 1996. Cell. 87: 1001–1014.

Serini G., Valdembri D., Bussolino F. Integrins and angiogenesis: a sticky business. 2006. Exp. Cell Res. 312: 651–658.

Shimizu T., Miyamoto A. Progesterone induces the expression of vascular endothelial growth factor (VEGF) 120 and Flk-1, its receptor, in bovine granulosa cells. 2007. Anim. Reprod. Sci. 102: 228–237.

Stasko S.E., Wagner G.F. Possible roles for stanniocalcin during early skeletal patterning and joint formation in the mouse. 2001. J. Endocrinol. 171: 237-248.

Staton C.A., Reed M.W., Brown N.J. A critical analysis of current in vitro and in vivo angiogenesis assays. 2009. Int. J. Exp. Pathol. 90: 195-221.

Struman I., Bentzien F., Lee H., Mainfroid V., D'angelo G., Goffin V., Weiner R. I., Martial J. A. Opposing actions of intact and N-terminal fragments of the human prolactin/growth hormone family members on angiogenesis: An efficient mechanism for the regulation of angiogenesis. 1999. Proc. Natl. Acad. Sci. USA. 96: 1246–1251.

Takahashi H., Shibuya M. The vascular endothelial growth factor (VEGF)/ VEGF receptor system and its role under physiological and pathological conditions. 2005. Clin. Sci. 109: 227-241.

Tamanini C., Basini G., Grasselli F., Tirelli M. Nitric oxide and the ovary. 2002. J. Anim. Sci. 81: 1-7.

Tamanini C., De Ambrogi M. Angiogenesis in developing follicle and corpus luteum. 2004. Reprod. Domestic Anim. 39: 206–216.

Tay C.C., Glasier A.F., McNeilly A.S. The 24 h pattern of pulsatile luteinizing hormone, follicle stimulating hormone and prolactin release during the first 8 weeks of lactational amenorrhoea in breastfeeding women. 1992. Hum. Reprod. 7: 951-958.

Thomas F.H., Wilson H., Silvestri A., Fraser H.M. Thrombospondin-1expression is increased during follicular atresia in the primate ovary. 2008. Endocrinology. 149: 185–192.

Ucuzian A.A., Gassman A.A., East A.T., Greisler H.P. Molecular Mediators of Angiogenesis. 2010. J. Burn Care Res. 31:158–175.

Ueda E., Ozerdem U., Chen Y.H., Yao M., Huang K.T., Sun H., Martins-Green M., Bartolini P., Walker A.M. A molecular mimic demonstrates that phosphorylated human prolactin is a potent anti-angiogenic hormone. 2006. Endocr. Relat. Cancer. 13: 95-111.

Valko M., Leibfritz D., Moncol J., Cronin M.T.D., Mazur M., Telser J. Free radicals and antioxidants in normal physiological functions and human disease. 2007. Int. J. Biochem. Cell Biol. 39: 44-84.

Van Wezel I.L, Umapathysivam K., Tilley W.D., Rodgers R.J. Immunohistochemical localization of basic fibroblast growth factor in bovine ovarian follicles. 1995. Mol. Cell. Endocrinol. 115: 133–140.

Varghese R., Wong C.K.C., Deol H., Wagner G. F., DiMattia G.E. Comparative analysis of mammalian stanniocalcin genes. 1998. Endocrinology. 139: 4714-4725.

Varinska L., Mirossay L., Mojzisova G., Mojzis J. Antiangogenic effect of selected phytochemicals. Pharmazie. 2010. 65: 57-63.

Wan C., Shao J., Gilbert S., Riddle R.C., Long F., Johnson R. S., Schipani E., Clemens T.L.. Role of HIF-1α in skeletal development. 2010. Ann. N.Y. Acad. Sci. 1192: 322-326.

Wang H., Copeland N. G., Gilbert D. J., Jenkins N. A. , Tessier-Lavigne M. Netrin-3, a mouse homolog of human NTN2L, is highly expressed in sensory ganglia and shows differential binding to netrin receptors. 1999. J. Neurosci. 19: 4938–4947.

Wang X., Howell C.P., Chen F., Yin J., Jiang Y. Gossypol--a polyphenolic compound from cotton plant. 2009. Adv. Food Nutr. Res. 58: 215-263.

Wary K.K., Thakker G.D., Humtsoe J.O., Yang J. Analysis of VEGF responsive genes involved in the activation of endothelial cells. 2003. Mol. Cancer. 9: 2-25.

Wilson B.D., Ii M., Won Park K., Suli A., Sorensen L.K., Larrieu-Lahargue F., Urness L.D., Suh W., Asai J., Kock G.A.H., Thorne T., Silver M., Thomas K.R., Chien C., Losordo D.W., Li D.Y. Netrins promote developmental and therapeutic angiogenesis. 2006. Science. 313: 640-644.

Wood J.R., Strauss J.F. Multiple signal transduction pathways regulate ovarian steroidogenesis. 2002. Rev. Endocr. Metab. Disord. 3: 33–46.

Yang M.Y., Fortune J.E. Vascular endothelial growth factor stimulates the primary to secondary follicle transition in bovine follicles in vitro. 2007. Mol. Reprod. Dev. 74: 1095–1104.

Yurchenco P.D., Wadsworth W.G. Assembly and tissue functions of early embryonic laminins and netrins. Curr. Opin. 2004. Cell Biol. 16: .572-579.

Zhang J., Alfonso P., Thotakura N.R., Su J., Buergin M., Parmelee D., Collins A.W., Oelkuct M., Gaffney S., Gentz S., Radman D.P., Wagner G.F., Gentz R. Expression, purification, and bioassay of human stanniocalcin from baculovirus-infected insect cells and recombinant CHO cells. 1998. Protein. Expr. Purif. 12: 390-398.

Zhang K., Lindesberg P.J., Tatlisumak T., Kaste M., Olsen H.S., Andersson L.C. Stanniocalcin: a molecular guard of neurons during cerebral ischemia. 2000. Proc. Natl. Acad. Sci. 97: 3637-3642.

Zhang Q., Tang X., Lu Q.Y., Zhang Z.F., Brown J., Le A.D. Resveratrol inhibits hypoxia-induced accumulation of hypoxia-inducible factor-1 alpha and VEGF expression in human tongue squamous cell carcinoma and hepatoma cells. 2005. Mol. Cancer Ther. 4: 1465-1474.

Zlot C., Ingle G., Hongo J., Yang S., Sheng Z., Schwall R., Paoni N., Wang F., Peale F.V., Gerritsen M.E. Stanniocalcin 1 is an autocrine modulator of endothelial angiogenic responses to hepatocyte growth factor. 2003. J. Biol. Chem. 278: 47654-47659.

ALLEGATO A

"Expression and localization of stanniocalcin 1 in swine ovary".

General and Comparative Endocrinology. 2010.
166: 404-408. doi:10.1016/j-ygcen.2009.12.013.

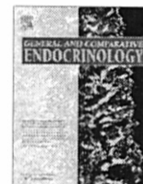
YGCEN 10533
28 December 2009
ARTICLE IN PRESS
No. of Pages 6, Model 5G

General and Comparative Endocrinology xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Expression and localization of stanniocalcin 1 in swine ovary

G. Basini [a,*], L. Baioni [a], S. Bussolati [a], S. Grolli [b], L.H. Kramer [c], G.F. Wagner [d], F. Grasselli [a]

[a] Sezione di Fisiologia Veterinaria, Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti, Italy
[b] Sezione di Biochimica Veterinaria, Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti, Italy
[c] Sezione di Parassitologia e Malattie Parassitarie, Dipartimento di Salute Animale, Via del Taglio 10 Università degli Studi di Parma, 43126 Parma, Italy
[d] Department of Physiology and Pharmacology, Faculty of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada N6A 5C1

ARTICLE INFO

Article history:
Received 26 October 2009
Revised 10 December 2009
Accepted 16 December 2009
Available online xxxx

Keywords:
Hypoxia
Ovary
Swine
Stanniocalcin 1

ABSTRACT

Stanniocalcin 1 (STC 1) is a glycoprotein involved in mineral homeostasis and was first identified in fish. Its mammalian homologue has been implicated in the regulation of various biological processes, including angiogenesis and steroidogenesis both of which are fundamental events in ovarian function. Interestingly, the highest level of STC 1 expression in mammals occurs in ovarian tissue but no information is available on swine species. Therefore, the present study was undertaken to investigate the expression and the immunolocalization of STC 1 in swine ovary. In addition, we evaluated whether swine granulosa cells synthesize STC 1 and its possible modulation by hypoxia, a physiological condition in ovarian follicle growth.

Our data show STC 1 for the first time in swine ovary; moreover, we demonstrate STC 1 production by granulosa cells, both in basal condition and in response to oxygen deprivation. The latter is suggestive of a potential modulatory role for STC 1 in hypoxia-driven angiogenesis.

© 2009 Published by Elsevier Inc.

1. Introduction

Stanniocalcin 1 (STC 1) is a glycoprotein hormone that was first identified in fish as being secreted by the corpuscles of Stannius, an organ unique to bony fish. Blood levels of STC 1 increase in response to elevated serum calcium and act on gills, kidneys and gut to regulate calcium absorption and phosphate excretion (Gerritsen and Wagner, 2005). In 1995 Chang et al., on the basis of its sequence homology with fish, isolated the first mammalian STC 1 cDNA clone from a human cell line. The hormone was subsequently found to be conserved in other mammalian species such as mouse (Chang et al., 1996), cow (Paciga et al., 2002) and sheep (Song et al., 2006). Though STC 1 gene expression is widespread in mammalian tissues, by far the highest level of expression is found in the ovaries (Varghese et al., 1998). Unlike its well defined role in regulating serum calcium in fish, little is known about STC 1 function in mammals. Interestingly, STC 1 has been demonstrated to play a role in steroidogenesis (Paciga et al., 2003) and angiogenesis (Chakraborty et al., 2007), fundamental processes in ovarian function (Basini et al., 2005, 2007, 2008a). On these bases, the present study was designed to investigate, for the first time, the expression and the immunolocalization of STC 1 in swine ovary. Moreover, our purpose was also to verify whether STC 1 is produced by granulosa cells and is present in swine follicular fluids. Finally, since a reduction in oxygen content physiologically takes place in ovarian follicles (Basini et al., 2004; Bianco et al., 2005) we also evaluated whether swine granulosa cell STC 1 production is modulated by hypoxic culture conditions.

2. Materials and methods

All reagents used in this study were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

2.1. Collection of ovaries

Swine ovaries were collected at a local slaughterhouse from Large White cross-bred gilts, placed into cold PBS (4 °C) supplemented with penicillin (500 IU/ml), streptomycin (500 μg/ml) and amphotericin B (3.75 μg/ml), maintained in a freezer bag and transported to the laboratory within 1 h.

2.2. Immunohistochemistry

Ovaries were placed in 10% buffered formalin and paraffin embedded. Following routine processing, 6-μm thick sections were placed on polylysine-treated slides and stained using a polyclonal rabbit antihuman STC 1 antiserum well characterized in terms of its specificity for STC 1 (Varghese et al., 1998) and previously validated in the swine (Song et al., 2009) according to the method described in Deol et al. (2000) slightly modified. Briefly, sections

* Corresponding author. Fax: +39 0521 032770.
E-mail address: basini@unipr.it (G. Basini).

0016-6480/$ - see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.ygcen.2009.12.013

Please cite this article in press as: Basini, G., et al. Expression and localization of stanniocalcin 1 in swine ovary. Gen. Comp. Endocrinol. (2009), doi:10.1016/j.ygcen.2009.12.013

were blocked in 10% normal swine serum for 20 min and then the primary antibody was applied (1:1000 dilution) and incubated overnight at 4 °C. At the end of incubation, slides were washed in PBS and then incubated with biotinylated secondary antibody for 15 min. After three 5-min washes in PBS, a complex of streptavidin–peroxidase (LSAB® + SYSTEM-HRP, Dako, Glostrup, Denmark) was applied for 15 min. Positive reactions were revealed by AEC (Dako, Glostrup, Denmark). Sections were then washed three times for 5 min with PBS and then counterstained with Gill's hematoxylin for 2 min. Control sections were subjected to the same procedure, except that diluted swine serum replaced the first antibody.

2.3. Follicles

Follicles were dissected from the ovaries and grouped on the basis of their diameter in small (<3 mm), medium (3–5 mm) and large (>5 mm) according to Basini et al. (2008b). Each follicle was cut open, oocytes were recovered and the remaining cumulus cells were removed by repeated pipetting with fine bore glass Pasteur pipettes with different diameters following the method described by Braga et al. (2007). Granulosa cells were separated from the follicles using a siliconized Pasteur pipette centrifuged at 300g for 10 min and at the end total RNA was extracted. Theca tissue was peeled from each follicle using fine forceps, frozen in liquid nitrogen and powdered in a mortar.

2.4. RNA extraction and semiquantitative RT-PCR

Total RNA was extracted from oocytes, granulosa and theca preparation using Nucleospin® RNA II (Macherey–Nagel GmbH, Duren, Germany) according to the manufacturer's instructions. Total RNA was quantified by absorbance at 260 nm (Gen Quant Pro, Amersham Biosciences, Freiburg, Germany). Total RNA (2 µg) was reverse transcribed with Ready-to-Go You Prime First-Strands Beads (Amersham Biosciences, Freiburg, Germany). STC 1 cDNA was PCR co-amplified with porcine β-actin (pACT) (Staszkiewicz et al., 2007; Shang et al., 2009) using two different primer pairs for the target genes. The constitutive pACT gene was used as an internal positive control to normalize the products of the amplification reaction. For STC 1, the sense primer (5′-TGATCAGTGCTT CTGCAACC-3′) and antisense primer (5′-TCACAGTCCAGTAGGC TTCG-3′) were derived from the bovine STC 1 mRNA coding sequence (GenBank Accession No. NM_176669; Song et al., 2006). Variability in mRNA amounts was assessed by amplifying swine actin using the primers pACT sense (5′-GAG ACC TTC AAC ACG CCG-3′) and pACT antisense (5′-GGA AGG TGG ACA GCG AGG-3′) (MWG Biotec, Ebersberg, Germany). An aliquot (5 µl) of the cDNA template was amplified by PCR using 1 µl (25 mU) *Taq* polymerase (Fermentas, Hannover, MD, USA) in 50 µl PCR buffer containing 1 µl of 10 mM dNTP mix (Fermentas), 0.3 µM for both pACT primers and 0.7 µM for both STC 1 primers. Amplification was carried out using the thermal cycler PTC-100 Peltier (MJ Research, San Francisco, CA, USA). After an initial denaturation step for 5 min at 95 °C, target cDNA was amplified for 2 cycles with a denaturation step at 95 °C for 30 s, annealing at 57 °C for 30 s (−1 °C in the second cycle), elongation at 72 °C for 30 s, and for 30 cycles with a denaturation step at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s. Reactions were terminated with a final elongation at 72 °C for 15 min. The PCR products were separated on 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) and visualized under UV light. Digital images were captured by Power Shot A610 (Canon, Tokyo, Japan). Quantification of band intensity was performed with NIH Image software Scion Image Beta 4.02 (Scion Corporation, MA, USA, http://rsb.info.nih.gov/nih-image/). Target gene mRNA abundance was expressed relative to pACT mRNA abundance. Semiquantitative PCR was validated by sequencing STC 1 amplicons (MGW Biotech, Ebersberg, Germany) after purification (Nucleospin, Machery Nagel, Duren).

2.5. Follicular fluid collection and processing

Follicular fluid was collected with a 26-gauge needle from follicles classified as mentioned above. The follicular aspirates were centrifuged (300g; 10 min) to quickly separate the fluid from the cell fraction. The supernatants were kept freezed until STC 1 assay.

2.6. Granulosa cell collection and hypoxia treatment

Ovaries were repeatedly washed with PBS and ethanol (70%). Granulosa cells were aseptically harvested by aspiration of large follicles (>5 mm) with a 26-gauge needle, released in medium containing heparin (50 IU/ml), centrifuged for pelleting and then treated with 0.9% prewarmed ammonium chloride at 37 °C for 1 min to remove red blood cells. Cell number and viability were estimated using a haemocytometer under a phase contrast microscope after vital staining with trypan blue (0.4%) of an aliquot of the cell suspension. Cells were seeded in DMEM/Ham's F12 supplemented with sodium bicarbonate (2.2 mg/ml), bovine serum albumin (BSA 0.1%), penicillin (100 IU/ml), streptomycin (100 µg/ml), amphotericin B (2.5 µg/ml), selenium (5 ng/ml) and transferrin (5 µg/ml). Once seeded in 24-well plates (10^6/ml), cells were incubated at 37 °C under humidified atmosphere (5% CO_2) for 24 h and then subjected for 18 h to normoxia (19% O_2), hypoxia (5% O_2) or anoxia (1% O_2). Anoxia was achieved employing an Anaerocult® A mini while hypoxia was obtained by means of an Anaerocult® C mini (Merck KgaA, Darmstadt, Germany). In both cases the system consisted of plastic pouches and a paper gas generating sachet (Basini et al., 2004).

2.7. STC 1 assay

STC 1 content in follicular fluid and granulosa culture media was quantified by RIA. The assay employs a polyclonal antibody produced as described by De Niu et al. (2000) to recombinant human STC and purified human STC for tracer and standard. The assay has a lower limit of detection of 0.2 ng/ml.

2.8. Statistical analysis

Immunohistochemistry and STC 1 expression experiments were repeated at least five times. STC 1 quantification was performed on 20 follicular fluids for each follicle class and granulosa cell cultures were repeated four times with four replicates for each treatment. Experimental data are presented as means ± SEM; statistical differences were calculated by ANOVA using Statgraphics package (STSC Inc., Rockville, MD, USA). When significant differences were found means were compared by Scheffè's *F* test.

3. Results

3.1. Immunohistochemistry

The most remarkable feature was the intense STC 1 immunoreactivity observed in the oocyte cytoplasm of both preantral and antral follicles (Fig. 1(A) and (B)). Weak staining was also evident in the granulosa and theca layer of antral follicles (medium and large) (Fig. 1(C)) and in the corpus luteum (Fig. 1(D)).

Please cite this article in press as: Basini, G., et al. Expression and localization of stanniocalcin 1 in swine ovary. Gen. Comp. Endocrinol. (2009), doi:10.1016/j.ygcen.2009.12.013

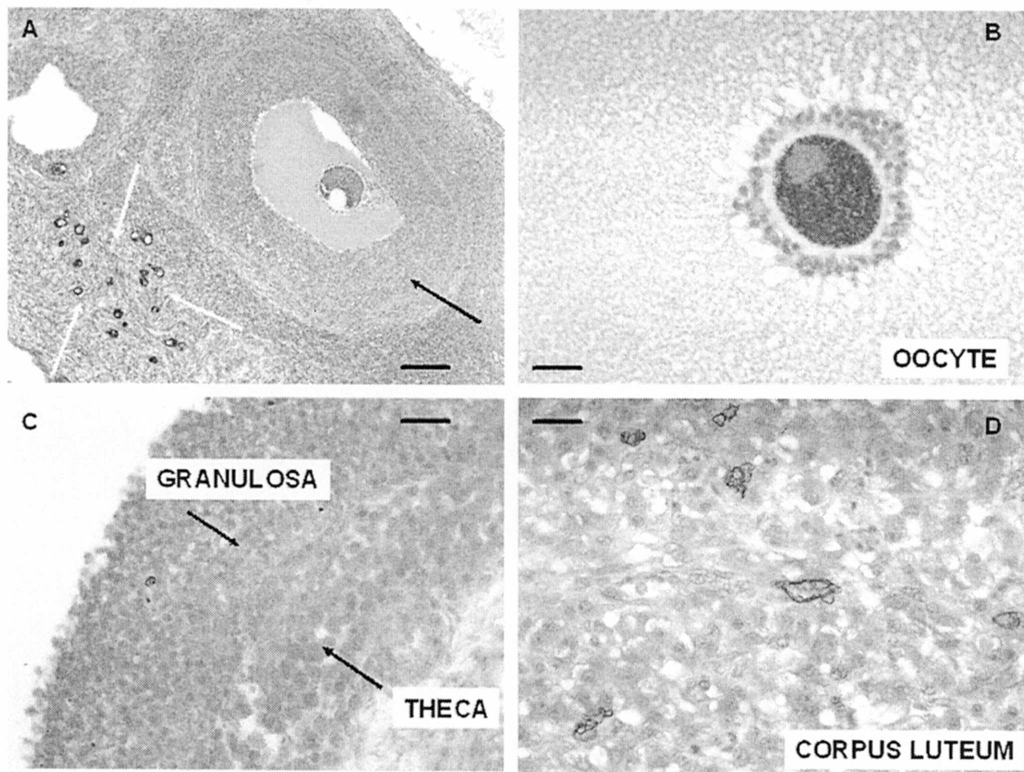


Fig. 1. Immunohistochemical localization of STC 1 in swine ovary. (A) Medium follicle (black arrow) and preantral follicles (white arrows) 10×. Bar, 200 μm. (B) Oocyte 40×. Bar, 60 μm. (C) Large follicle wall 40×. Bar, 60 μm. (D) Corpus luteum 40×. Bar, 60 μm.

193 *3.2. STC 1 expression*

194 Amplified DNA (length 466 bp) was sequenced and its identity
195 confirmed running a blast against the reference sequence of por-
196 cine mRNA (GenBank Accession No. EU086592; Song et al.,
197 2009). Furthermore, a blast against mammalian protein sequences
198 confirm that porcine STC 1 is highly conserved. In particular, it
199 shows a 95% identity to bovine protein, 94% identity to human
200 and canine proteins, 93% identity to equine protein and 50% iden-
201 tity to rainbow trout protein.

202 STC 1 expression was evident in the oocytes and in the granu-
203 losa and theca layers from different sized follicles.

204 In particular, oocytes derived from small, medium and large fol-
205 licles showed STC 1 expression without any significant difference
206 among them (Fig. 2).

207 In the theca layers, the highest expression was found in small
208 follicles ($p < 0.05$) which decrease ($p < 0.05$) during follicle growth.
209 In contrast, STC 1 levels in granulosa cells were directly related
210 ($p < 0.05$) with follicle diameter since they significantly increased
211 during follicle growth (Fig. 3).

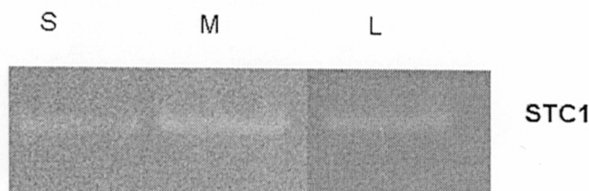


Fig. 2. STC 1 expression in swine oocytes collected from follicles classified as small (S), medium (M) and large (L). The representative ethidium bromide-stained gel shows STC 1 amplicons from 30 oocytes from each kind of follicles.

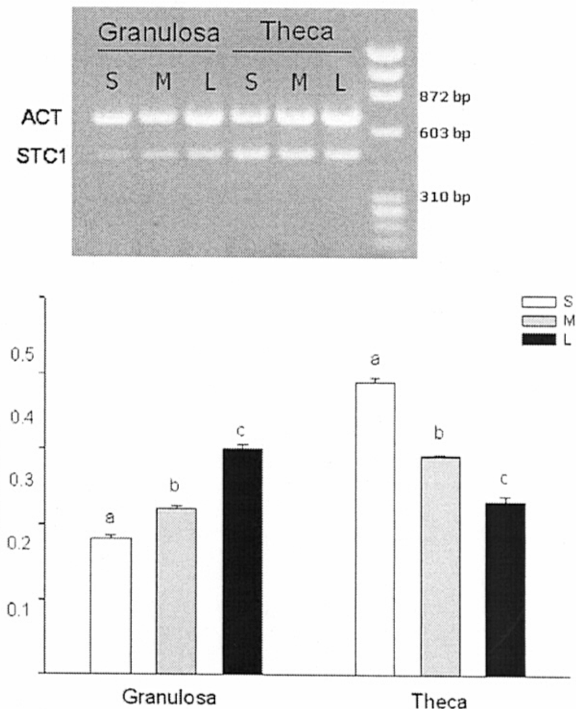


Fig. 3. Relative quantification of STC 1 in granulosa and theca cells from follicles classified as small (S), medium (M) and large (L). Steady-state mRNA levels were measured by semiquantitative RT-PCR and expressed relative to the housekeeping gene pACT. The representative ethidium bromide-stained gel shows pACT and STC 1 amplicons from at least three follicles. Data are expressed as means ± SEM. Different letters indicate significant difference ($p < 0.05$) in the same cell layer.

Please cite this article in press as: Basini, G., et al. Expression and localization of stanniocalcin 1 in swine ovary. Gen. Comp. Endocrinol. (2009), doi:10.1016/j.ygcen.2009.12.013

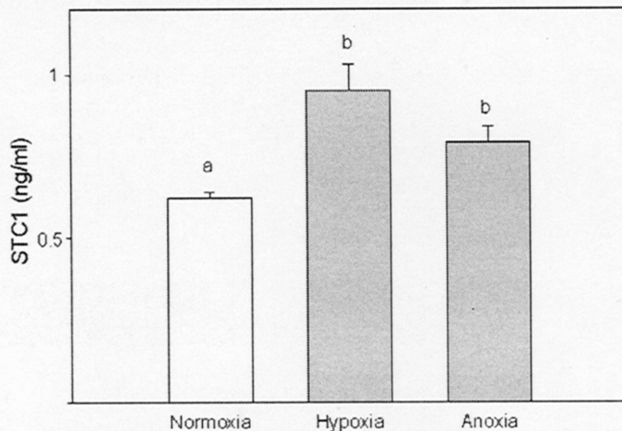


Fig. 4. Effect of hypoxia and anoxia on STC 1 production (ng/ml) by swine granulosa cells. Granulosa cell cultures were repeated four times with four replicates for each treatment. Data are expressed as means ± SEM. Different letters indicate significant difference ($p < 0.05$).

3.3. *STC 1 quantification in follicular fluid and in granulosa culture media*

STC 1 was present in swine follicular fluid at a mean concentration of 8.4 ± 0.9 (ng/ml; mean ± SEM). No differences were detected in the fluids of different size follicles.

Cultured swine granulosa cells appeared to produce STC 1. Moreover, hypoxia and anoxia significantly increased STC 1 output ($p < 0.01$). No significant differences were detected between the two different oxygen deprivation tested (Fig. 4).

4. Discussion

Since 1995, when Chang et al. discovered mammalian STC 1 (Chang et al., 1995), several studies have been aimed to unravel the biological significance of this hormone in mammals.

Analyses of STC 1 gene expression showed that, at least in the mouse, the ovary displays the highest levels of STC 1 mRNA (Varghese et al., 1998; Deol et al., 2000). This observation would suggest a potential involvement of STC 1 in regulating ovarian function.

To the best of our knowledge, the present study identifies STC 1 in swine ovary for the first time. In particular, we have demonstrated that STC 1 protein is present in swine ovarian follicles, is present in follicular fluid and is produced by granulosa cells.

As for gene expression, transcription was detected in the theca layer from antral follicles. This is in agreement with the findings in the mouse (Varghese et al., 1998), rat and bovine (Paciga et al., 2002). Moreover, our data showed that STC 1 expression in theca layer cells decrease during follicle growth. In addition, our experiments showed that STC 1 is also expressed in granulosa cells, with an increasing trend during follicular development. This result is surprising as STC 1 expression in the mouse, rat and bovine ovary has been reported to be confined to theca-interstitial cells, with no apparent expression elsewhere (Paciga et al., 2002; Varghese et al., 1998). At the moment, there is no information concerning STC 1 expression in granulosa cells in other mammalian species. In particular, as shown in the mouse (Varghese et al., 1998; Deol et al., 2000) our study showed that STC 1 protein is strongly concentrated and its gene is also expressed in the swine oocyte. These findings are partly in contrast with the sequestering hypothesis formulated in the kidney by Wong et al. (2002) whereby STC 1 appeared to be synthesized and released by one cell type and heavily sequestered by its target cells. This hypothesis was further reinforced by the findings obtained in the ovary (Deol et al., 2000) and in the developing mouse embryo (Stasko and Wagner, 2001) and it was assumed that the process was receptor-mediated. However, the underlying purpose for the sequestering has not been clarified yet.

These data, along with the evidence of STC 1 presence in swine follicular fluid, lead us to suppose that STC 1 may be involved in different aspects of ovarian follicle maturation. The exact role of STC 1 in ovarian dynamics is still mostly unknown, even if experimental evidence suggest its involvement in the regulation of follicular development (Luo et al., 2004). Since lipid and cholesterol storage droplets represent preferential subcellular targets of STC 1 (Paciga et al., 2003), a modulation of the steroidogenetic pathway by this hormone could be assumed (Baioni et al., 2009).

Interestingly, earlier experimental data have also demonstrated a role of STC 1 in the angiogenic process (Wary et al., 2003; Zlot et al., 2003; Holmes and Zachary, 2008; Klein et al., 2009; Basini et al., 2009). Therefore, the presence of STC 1 in the ovarian follicle in this context may be relevant, since neovascularization is closely associated with oocyte growth and development (Bianco et al., 2005; Basini et al., 2007, 2008a). In particular, we have previously demonstrated that, among the various triggers of angiogenesis, low oxygen tension appears as a particularly important stimulus (Basini et al., 2004). The present data demonstrate that STC 1 secretion by granulosa cells is increased by oxygen deprivation. Moreover, several authors (Yeung et al., 2005; Westberg et al., 2007) have demonstrated that the activation of STC 1 expression is increased in cells exposed to hypoxic stress. These results are therefore suggestive of a potential role for STC 1 in ovarian follicle physiology where progressive hypoxia is established during follicle growth.

Further studies are in due course in our laboratory to get an insight on the physiological significance of STC 1 in swine ovarian follicle.

5. Uncited reference

Horiuchi et al. (2002). Q1

Acknowledgment

This study was supported by a MIUR PRIN grant.

References

Baioni, L., Basini, G., Bussolati, S., Grasselli, F., 2009. Stanniocalcin 1 is a potential physiological modulator of steroidogenesis in the swine ovarian follicle. Vet. Res. Commun. 33, 73–76.
Basini, G., Bianco, F., Grasselli, F., Tirelli, M., Bussolati, S., Tamanini, C., 2004. The effects of reduced oxygen tension on swine granulosa cell. Regul. Pept. 15, 69–75.
Basini, G., Bianco, F., Grasselli, F., 2005. Epigallocatechin-3-gallate from green tea negatively affects swine granulosa cell function. Domest. Anim. Endocrinol. 28, 243–256.
Basini, G., Bussolati, S., Santini, S.E., Bianchi, F., Careri, M., Mangia, A., Musci, M., Grasselli, F., 2007. Antiangiogenesis in swine ovarian follicle: a potential role for 2-methoxyestradiol. Steroids 72, 660–665.
Basini, G., Bussolati, S., Santini, S.E., Bianchi, F., Careri, M., Mangia, A., Musci, M., Grasselli, F., 2008a. Hydroxyestrogens inhibit angiogenesis in swine ovarian follicles. J. Endocrinol. 199, 127–135.
Basini, G., Bussolati, S., Santini, S.E., Grasselli, F., 2008b. Reactive oxygen species and anti-oxidant defences in swine follicular fluids. Reprod. Fertil. Dev. 20, 269–274.
Basini, G., Bussolati, S., Santini, S.E., Grasselli, F., 2009. Stanniocalcin, a potential ovarian angiogenesis regulator, does not affect endothelial cell apoptosis. Ann. NY Acad. Sci. 1171, 94–99.
Bianco, F., Basini, G., Grasselli, F., 2005. Angiogenic activity of swine granulosa cells: effects of hypoxia and vascular endothelial growth factor Trap R1R2, a VEGF blocker. Domest. Anim. Endocrinol. 28, 308–319.
Braga, D.P., Pasqualotto, F.F., Maraschi, C., Sonetti, T.C., Rodrigues, D., Iaconelli Jr., A., Borges Jr., E., 2007. Use of pig oocytes for training new professionals in human assisted reproduction laboratories. Fertil. Steril. 88, 1408–1412.

Please cite this article in press as: Basini, G., et al. Expression and localization of stanniocalcin 1 in swine ovary. Gen. Comp. Endocrinol. (2009), doi:10.1016/j.ygcen.2009.12.013

Chakraborty, A., Brooks, H., Zhang, P., Smith, W., McReynolds, M.R., Hoying, J.B., Bick, R., Truong, L., Poindexter, B., Lan, H., Elbjeirami, W., Sheikh-Hamad, D., 2007. Stanniocalcin-1 regulates endothelial gene expression and modulates transendothelial migration of leukocytes. Am. J. Physiol. Renal Physiol. 292, 895–904.

Chang, A.C., Dunham, M.A., Jeffrey, K.J., Reddel, R.R., 1996. Molecular cloning and characterization of mouse stanniocalcin cDNA. Mol. Cell. Endocrinol. 124, 185–187.

Chang, A.C., Janosi, J., Hulsbeek, M., de Jong, D., Jeffrey, K.J., Noble, J.R., Reddel, R.R., 1995. A novel human cDNA highly homologous to the fish hormone stanniocalcin. Mol. Cell. Endocrinol. 112, 241–247.

De Niu, P., Radman, D.P., Jaworski, E.M., Deol, H., Gentz, R., Su, J., Olsen, H.S., Wagner, G.F., 2000. Development of a human stanniocalcin radioimmunoassay: serum and tissue hormone levels and pharmacokinetics in the rat. Mol. Cell. Endocrinol. 162, 131–144.

Deol, H.K., Varghese, R., Wagner, G.F., Dimattia, G.E., 2000. Dynamic regulation of mouse ovarian stanniocalcin expression during gestation and lactation. Endocrinology 141, 3412–3421.

Gerritsen, M.E., Wagner, G.F., 2005. Stanniocalcin: no longer just a fish tale. Vitam. Horm. 70, 105–135.

Holmes, D.I., Zachary, I.C., 2008. Vascular endothelial growth factor regulates stanniocalcin-1 expression via neuropilin-1-dependent regulation of KDR and synergism with fibroblast growth factor-2. Cell. Signal. 20, 569–579.

Horiuchi, A., Imai, T., Shimizu, M., Oka, K., Wang, C., Nikaido, T., Konishi, I., 2002. Hypoxia-induced changes in the expression of VEGF, HIF-1 alpha and cell cycle-related molecules in ovarian cancer cells. Anticancer. Res. 22, 2697–2702.

Klein, D., Demory, A., Peyre, F., Kroll, J., Géraud, C., Ohnesorge, N., Schledzewski, K., Arnold, B., Goerdt, S., 2009. Wnt2 acts as an angiogenic growth factor for non-sinusoidal endothelial cells and inhibits expression of stanniocalcin-1. Angiogenesis (Epub ahead of print).

Luo, C.W., Kawamura, K., Klein, C., Hsueh, A.J., 2004. Paracrine regulation of ovarian granulosa cell differentiation by stanniocalcin (STC) 1: mediation through specific STC1 receptors. Mol. Endocrinol. 18, 2085–2096.

Paciga, M., Watson, A.J., DiMattia, G.E., Wagner, G.F., 2002. Ovarian stanniocalcin is structurally unique in mammals and its production and release are regulated through the luteinizing hormone receptor. Endocrinology 143, 3925–3934.

Paciga, M., McCudden, C.R., Londos, C., DiMattia, G.E., Wagner, G.F., 2003. Targeting of big stanniocalcin and its receptor to lipid storage droplets of ovarian steroidogenic cells. J. Biol. Chem. 278, 49549–49554.

Shang, H., Yang, J., Liu, Y., Wei, H., 2009. Tissue distribution of CYP3A29 mRNA expression in Bama miniature pig by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Xenobiotica 39, 423–429.

Song, G., Bazer, F.W., Wagner, G.F., Spencer, T.E., 2006. Stanniocalcin (STC) in the endometrial glands of the ovine uterus: regulation by progesterone and placental hormones. Biol. Reprod. 74, 913–922.

Song, G., Dunlap, K.A., Kim, J., Bailey, D.W., Spencer, T.E., Burghardt, R.C., Wagner, G.F., Johnson, G.A., Bazer, F.W., 2009. Stanniocalcin 1 is a luminal epithelial marker for implantation in pigs regulated by progesterone and estradiol. Endocrinology 150, 936–945.

Stasko, S.E., Wagner, G.F., 2001. Stanniocalcin gene expression during mouse urogenital development: a possible role in mesenchymal–epithelial signalling. Dev. Dyn. 220, 49–59.

Staszkiewicz, J., Skowronski, M.T., Kaminski, T., Siawrys, G., Krazinski, B.E., Kusmider, M., Przala, J., Okrasa, S., 2007. Expression of proopiomelanocortin, proenkephalin and prodynorphin genes in porcine theca and granulosa cells. Anim. Reprod. Sci. 101, 97–112.

Varghese, R., Wong, C.K., Deol, H., Wagner, G.F., DiMattia, G.E., 1998. Comparative analysis of mammalian stanniocalcin genes. Endocrinology 139, 4714–4725.

Wary, K.K., Thakker, G.D., Humtsoe, J.O., Yang, J., 2003. Analysis of VEGF-responsive genes involved in the activation of endothelial cells. Mol. Cancer 9, 2–25.

Westberg, J.A., Serlachius, M., Lankila, P., Andersson, L.C., 2007. Hypoxic preconditioning induces elevated expression of stanniocalcin-1 in the heart. Am. J. Physiol. Heart Circ. Physiol. 293, 1766–1771.

Wong, C.K., Ho, M.A., Wagner, G.F., 2002. The colocalization of stanniocalcin protein mRNA and kidney cell markers in the rat kidney. J. Endocrinol. 173, 199–209.

Yeung, H.Y., Lai, K.P., Chan, H.Y., Mak, N.K., Wagner, G.F., Wong, C.K., 2005. Hypoxia-inducible factor-1-mediated activation of stanniocalcin-1 in human cancer cells. Endocrinology 146, 4951–4960.

Zlot, C., Ingle, G., Hongo, J., Yang, S., Sheng, Z., Schwall, R., Paoni, N., Wang, F., Peale Jr., F.V., Gerritsen, M.E., 2003. Stanniocalcin 1 is an autocrine modulator of endothelial angiogenic responses to hepatocyte growth factor. J. Biol. Chem. 278, 47654–47659.

Please cite this article in press as: Basini, G., et al. Expression and localization of stanniocalcin 1 in swine ovary. Gen. Comp. Endocrinol. (2009), doi:10.1016/j.ygcen.2009.12.013

ALLEGATO B

“Stanniocalcin 1 is a potential physiological modulator of steroidogenesis in the swine ovarian follicle”

Vet Res Commun. 2009. 33: S73-S76.
DOI:10.1007/s11259-009-9252-1.

AUTHOR'S PROOF JrnlID 11259_ArtID 9252_Proof# 1 - 25/06/2009

Vet Res Commun
DOI 10.1007/s11259-009-9252-1

ABSTRACT

Stanniocalcin 1 is a potential physiological modulator of steroidogenesis in the swine ovarian follicle

L. Baioni · G. Basini · S. Bussolati · F. Grasselli

© Springer Science + Business Media B.V. 2009

Keywords Estradiol 17β · Granulosa cells · Ovary · Progesterone · Stanniocalcin-1

Abbreviations

E2	estradiol 17β
P4	progesterone
STC1	Stanniocalcin 1

Introduction

Granulosa cells, which surround and nurse the oocyte up to its complete maturation, play a central role in the function of the ovarian follicle (Woodruff and Shea 2007). Steroidogenesis, namely the synthesis of E2 and P4, is undoubtedly one of the events characterizing ovarian follicle development (Palermo 2007). Therefore, the studies aimed at unravelling the autocrine and/or paracrine mechanisms involved in the molecular regulation of these pathways deserve special attention. We have previously shown that swine granulosa cells express STC1 (Santini et al. 2007), a glycoproteic hormone identified in bony fish and subsequently demonstrated as a pleiotropic factor in mammals (Gerritsen and Wagner 2005). Recent data underline that STC1 is significantly involved in regulatory events in reproductive physiology (Li and Wong 2008). The exact role of the STC1 in ovarian dynamics is still mostly unknown, even if much experimental evidence suggests its involvement in the regulation of follicular development (Luo et al. 2004). Since lipid and cholesterol storage droplets represent preferential subcellular targets of STC1 (Paciga et al. 2003), a modulation of the steroidogenetic pathway by this hormone could be assumed. In

L. Baioni · G. Basini (✉) · S. Bussolati · F. Grasselli
Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti, Facoltà di Medicina Veterinaria, Università degli Studi di Parma, Via del Taglio 10, 43100 Parma, Italy
e-mail: basini@unipr.it

Results 51

E2 and P4 levels in the control group were 5.53±0.50 ng/ml (mean ± SEM) and 84.70± 8.90 ng/ml, respectively. E2 production was significantly inhibited ($p<0.001$) by STC1 treatment; no significant differences between the different concentrations tested was observed ($p<0.001$) (Fig. 1). On the contrary, P4 production was significantly stimulated ($p<0.05$) by STC1 without significant differences between treatment concentrations ($p <0.05$) (Fig. 2). 52–56

Discussion 57

The existence of STC1 in mammals was first demonstrated in 1995 by Chang and his colleagues (Chang et al. 1995): since then, several studies have attempted to clarify its biological role. Analysis of STC1 gene expression revealed that, at least in mice and man, the highest levels of STC1 mRNA are found in the ovary (Deol et al. 2000); this finding suggests a potential role of this peptide in the regulation of ovarian physiology. In a previous work (Santini et al. 2007), we documentated the expression of STC1 in swine ovarian follicle: the increased expression in the granulosa layer during follicular development would suggest STC1 involvement in follicle maturation. Granulosa cells have a pivotal role in this event, mainly through the synthesis of hormones that accomplish follicular growth. Present results indicate that STC1 treatment stimulates P4 and inhibits E2 production. Luo and colleagues (2004) have reported that STC1 inhibits P4 production from rat granulosa cells while E2 production is unaffected by the treatment with this hormone. These discrepancies could be due to differences in the experimental model: first of all, the effects could be species-specific; moreover, our data have been obtained in more differentiated granulosa cells. In addition, we have only evaluated steroid production in basal conditions while gonadotropin-induced steroidogenesis was studied in the model of Luo and coworkers. Finally, a further source of variability could be represented by the different concentrations tested. Taken together, we can point out that STC1 is a local stimulator of granulosa cell differentiation toward luteal cells: in fact, this process is characterized by a progressive shift from E2 to P4 production. STC1 is potentially involved in the modulation of this event, resulting from the differentiation process of the steroidogenic pathway (Stouffer 2004). 58–79

Acknowledgements This work was supported by MIUR-PRIN and FIL grants. 80

81

References 82

Chang A.C., Janosi J., Hulsbeek M., de Jong D., Jeffrey K.J., Noble J.R., Reddel R.R., 1995. A novel human cDNA highly homologous to the fish hormone stanniocalcin. *Molecular and Cellular Endocrinology*, **112**, 241–247. 83–85

Deol H.K., Varghese R., Wagner G.F., Dimattia G.E., 2000. Dynamic regulation of mouse ovarian stanniocalcin expression during gestation and lactation. *Endocrinology*, **141**, 3412–3421. 86–87

Gerritsen M.E. and Wagner G.F., 2005. Stanniocalcin: no just a fish tale. *Vitamins and Hormones*, **70**, 105–135. 88–89

Grasselli F., Baratta M., Tamanini C., 1993. Effects of GnRH analogue (buserelin) infused via osmotic minipumps on pituitary and ovarian activity of prepuberal heifers. *Animal Reproduction Science*, **32**, 153–161. 90–91

Li L. and Wong C.K., 2008. Effects of dexamethasone and dibutyryl cAMP on stanniocalcin-1 mRNA expression in rat primary Sertoli and Leydig cells. *Molecular and Cellular Endocrinology*, **283**, 96–103. 92–93

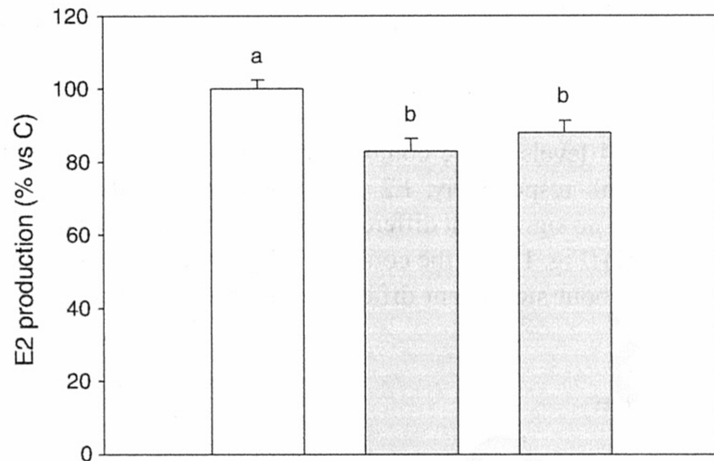


Fig. 1 STC1 effects on E2 production by granulosa cells. Data are expressed as mean ± SEM. Different letters indicate a significant ($p<0.05$) difference

order to verify this hypothesis, the present study was addressed at investigating the potential effects of STC1 on the steroidopoietic activity of swine granulosa cells. 37 38

Materials and methods 39

Swine ovaries were obtained from a slaughterhouse. Granulosa cells were harvested by aspiration from follicles >5 mm; 10^4 cells were seeded in 96-well plates in 200 µl of M199 supplemented with sodium bicarbonate (2.2 mg/ml), bovine serum albumin (0.1%), penicillin (100 UI/ml), streptomycin (100 µg/ml), amphotericin B (2.5 µg/ml), selenium (5 ng/ml), transferrin (5 µg/ml) and androstenedione (28 ng/ml) and treated with STC1 (Biovendor Laboratory Medicine Inc., Candler, USA) at the concentration of 5 and 50 ng/ml. Thereafter, cells were incubated at 37 C under a humidified atmosphere (5% CO_2) for 48 h. Culture media were then collected, frozen and stored at −20°C until P4 and E2 measurement by validated RIAs (Grasselli et al. 1993). Experimental data were analysed by means of ANOVA. Where significant differences were found ($p<0.05$), means were compared by Scheffè's F test for multiple comparison. 40 41 42 43 44 45 46 47 48 49 50

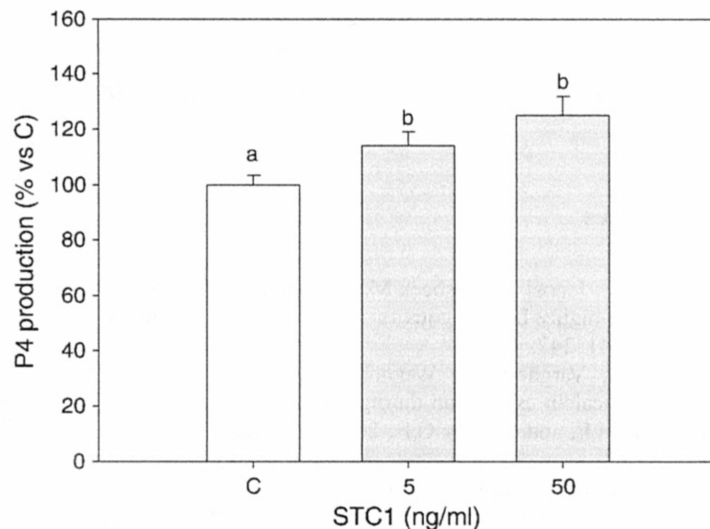


Fig. 2 STC1 effects on E2 production by granulosa cells. Data are expressed as mean ± SEM. Different letters indicate a significant ($p<0.05$) difference

Luo C.W., Kawamura K., Klein C., Hsueh A.J., 2004. Paracrine regulation of ovarian granulosa cell differentiation by stanniocalcin (STC) 1: mediation through specific STC1 receptors. *Molecular Endocrinology*, **18**, 2085–2096. 94 95 96

Paciga M., McCudden C.R., Londos C., DiMattia G.E., Wagner G.F., 2003. Targeting of big stanniocalcin and its receptor to lipid storage droplets of ovarian steroidogenic cells. *The Journal of Biological Chemistry*, **278**, 49549–49554. 97 98 99

Palermo R., 2007. Differential actions of FSH and LH during folliculogenesis. *Reproductive Biomedicine Online,* **15**, 326–337. 100 101

Santini S.E., Basini G., Bussolati S., Baioni L., Grolli S., Grasselli F., 2007. Expression of stanniocalcin-1 in swine ovarian follicle: dependence on follicular size. *Atti LXI Convegno Nazionale Società Italiana delle Scienze Veterinarie*, 87–88. 102 103 104

Stouffer R.L., 2004. The Functions and Regulation of Cell Populations Comprising the Corpus Luteum during the Ovarian Cycle. In: *The ovary*, E. Adashi and P.C.K. Leung (Eds). Elsevier Academic Press: S. Diego, 169–180. 105 106 107

Woodruff T.K. and Shea L.D., 2007. The role of the extracellular matrix in ovarian follicle development. *Reproductive Sciences*, **14,** 6–10. 108 109

110

UNCORRECTED PROOF

ALLEGATO C

"Stanniocalcin 1 affects redox status of swine granulosa cells".

Sottoposto alla rivista *Regulatory Peptides.*

1 # Stanniocalcin 1 affects redox status of swine granulosa cells

2 Laura Baioni, Giuseppina Basini*, Simona Bussolati, Francesca Grasselli

3 *Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli*
4 *Alimenti - Sezione di Fisiologia Veterinaria - Via del Taglio 10, Università degli Studi di*
5 *Parma, 43126 Parma, Italy*

6
7
8 ## A B S T R A C T
9

10 Stanniocalcin 1 (STC1) is a glycoprotein hormone expressed in different mammalian
11 tissues. In previous studies, we showed STC1 expression in swine ovarian follicles and we
12 demonstrated that STC1 may be a physiological regulator of follicular function. Since
13 reactive oxygen species (ROS) are important signal transducers in the ovary, the present
14 study was undertaken to investigate STC1 action on ROS generation and on the activity of
15 the major enzymatic and non-enzymatic scavengers in swine granulosa cells. Taken
16 together, our data show that STC1 modulates redox status in swine granulosa cells.

17

18 Keywords: granulosa cells, swine, Stanniocalcin 1, redox status , ovary

19
20
21
22
23
24
25
26

* Corresponding author. Fax: +39-0521-032770
E-mail address: basini@unipr.it (G. Basini)

1. Introduction

Stanniocalcin 1 (STC1) is a peptidic hormone firstly identified in bony fishes [1] as a regulator of calcium/phosphate homeostasis [2, 3]. The mammalian STC1 homologue is largely expressed in different tissues of many species [4-6], with the highest level in the ovary [7, 8]. STC1 regulatory effects on calcium and phosphate exchange are conserved from fish to mammals [9, 10]. In the latter, STC1 involvement has been also documented in a variety of biological processes such as apoptosis [11, 12], inflammation [13] and reproduction [14]. In particular, several lines of experimental support STC1 involvement in regulating endothelial functions and angiogenesis [15-17]. In a previous study, we have shown STC1 expression in swine ovarian follicles, its presence in follicular fluid and its production by granulosa cells [18]. In addition, we demonstrated that STC1 could be a physiological regulator of follicular function by modulating steroidogenesis [19] and by inhibiting the production of the main proangiogenic factor Vascular Endothelial Growth Factor (VEGF) by granulosa cells [20], thus suggesting an involvement of STC1 in ovarian angiogenesis fine tuning. Reactive oxygen species (ROS) have been previously linked to angiogenesis signalling mechanisms in the ovary [21, 22]. Moreover, in the ovary ROS may have a regulatory role in various processes such as folliculogenesis, ovarian steroid production, oocyte maturation, corpus luteum development and luteolysis [23]. Based on these findings, in order to get a deeper insight on the physiological significance of STC1 in swine ovarian follicle function and follicular angiogenesis we investigated its effect on the production of the main ROS species, namely superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) by swine ovarian granulosa cells. In addition, we also verified STC1 effects on the activity of the major enzymatic and non-enzymatic ROS detoxification systems in swine granulosa cells.

53 ## 2. Materials and methods

54 All reagents used in this study were obtained from Sigma (St. Louis, MO, USA) unless
55 otherwise specified.

56

57 *2.1 Granulosa cell collection*

58 Swine ovaries were obtained from a local slaughterhouse. The ovaries were placed into
59 cold PBS (4°C) supplemented with penicillin (500 IU/ml), streptomycin (500 μg/ml) and
60 amphotericin B (3.75 μg/ml), maintained in a cold bag and transported to the laboratory
61 within 1 h. After a series of washings with PBS and ethanol (70%) granulosa cells were
62 aseptically harvested by aspiration of large follicles (> 5 mm) with a 26-gauge needle and
63 released in medium containing heparin (50 IU/ml), centrifuged for 10 min at 400xg
64 pelleting and then treated with 0.9% prewarmed ammonium chloride at 37 °C for 1 min to
65 remove red blood cells. Cell number and viability were estimated using a haemocytometer
66 under a phase contrast microscope after vital staining with trypan blue (0.4%) of an
67 aliquot of the cell suspension. Cells were seeded in culture medium (CM) DMEM/F-12 +
68 GlutaMax-1 (Invitrogen, Carisbad, CA, USA) supplemented with sodium bicarbonate (2.2
69 mg/ml), bovine serum albumin (BSA 0.1%), penicillin (100 IU/ml), streptomycin (100 μg/ml),
70 amphotericin B (2.5 μg/ml), selenium (5 ng/ml) and transferrin (5 μg/ml). Once seeded, cells
71 were treated with 5 and 50 ng/ml of STC 1 (BioVendor Laboratory Medicine, Candler, NC)
72 and maintained for 48 h at 37 °C under humidified atmosphere (5% CO_2). This procedure
73 was identical for all experiments performed in this study.

74

75 *2.2 Granulosa cell proliferation*

76 Cell proliferation was evaluated by 5-bromo-2-deoxyuridine (BrdU) incorporation assay
77 test (Roche, Mannheim, Germany). Briefly, 10^4 cell/200 μl CM were seeded in 96-well plates
78 and treated as described above. After addition of 20 μl BrdU to each well during the last 4

79 h of culture, culture media were removed, and DNA denaturing solution was added in
80 order to improve the accessibility of the incorporated BrdU for antibody detection.
81 Thereafter, 100 μl anti-BrdU antibody were added to each well. After a 1.5-h incubation at
82 room temperature (21 °C), the immune complexes were detected by the subsequent
83 substrate reaction. The reaction product was quantified by measuring the absorbance at
84 450nm against 690 nm using Spectra ShellMicroplate reader (SLT Spectra, Milan, Italy). To
85 establish viable cell number, absorbance was related to a standard curve prepared by
86 culturing in quintuplicate granulosa cells at different plating densities (from 10^3 to 10^5/200
87 μl) for 48 h. The curve was repeated in four different experiments. The relationship between
88 cell number and absorbance was linear (r = 0.92).
89 Cell number/well was estimated from the resulting linear regression equation. The assay
90 detection limit was 10^3 cell/well, and the variation coefficient was less than 5%. The number
91 of cells obtained by this calculation was used for correcting redox status data.
92
93 *2.3. Reactive oxygen species*
94 *2.3.1 Superoxide anion (O_2^-)*
95 O_2^- production was evaluated by a WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-
96 tetrazolio]-1,3-benzene disulfonate) test (Roche, Mannheim, Germany). The assay is based
97 on the cleavage of the water-soluble tetrazolium salt, WST-1 to a yellow-orange, water
98 soluble formazan. 10^4 cells/200 μl CM were seeded in 96-well plates. 20 μl WST-1 were
99 added to cells during the last 4 h of incubation and absorbance was then determined using
100 a Spectra Shell Microplate reader at a wavelength of 450 nm against 620 nm [24].
101
102 *2.3.2 Hydrogen peroxide (H_2O_2)*
103 Hydrogen peroxide (H_2O_2) production was measured by an Amplex Red Hydrogen
104 Peroxide Assay Kit (Molecular Probes, PoortGebouw, The Netherlands); the Amplex Red

105 reagent reacts with H_2O_2 to produce resorufin, an oxidation product. Briefly, 2 × 10^5
106 cells/200 µl CM were seeded in 96-well plates. After incubation with treatments, plates were
107 centrifuged for 10 min, at 400 × g, then the supernatants were discarded and granulosa
108 cells were lysed by adding cold Triton 0.5% + phenylmethylsulphonyl fluoride (PMSF) in PBS
109 (200 µl/well) and incubating on ice for 30 min. The test was performed on cell lysates and
110 read against a standard curve of H_2O_2 ranging from 0.39 to 25 µM. A Spectra Shell
111 microplate reader set to read 540 nm emission was used to quantify the reaction product.
112
113 *2.4 Scavenging enzymatic activity*
114 The procedure performed to obtain granulosa cells lysates is the same for all the
115 scavenger enzymes.
116 Briefly, 2 ×10^5 cells/200µl CM were seeded in 96-well plates and incubated in conditions
117 described above . After centrifugation for 10 min at 400 x g, the surnatants were discarded
118 and granulosa cells were lysed adding cold Triton 0.5% + phenylmethylsulphonyl fluoride
119 (PMSF) in PBS (200 µl/well) and incubating on ice for 30 min
120
121 *2.4.1 Superoxide dismutase activity*
122 SOD activity was determined by a SOD Assay Kit (Dojindo Molecular Technologies,
123 Japan). Cell lysates were tested without dilution. A standard curve of SOD ranging from
124 0.156 to 20 U/ml was prepared. The assay measures formazan produced by the reaction
125 between tetrazolium salt (WST-1) and superoxide anion (O_2^-), generated adding xantine
126 oxidase. Endogenous SOD activity is related to the remaining O_2^-. The absorbance was
127 determined with a Spectra Shell Microplate Reader reading at a wavelength of 450 nm
128 against 620 nm.
129
130 *2.4.2 Peroxidase activity*

131 Peroxidase activity was measured by an Amplex Red Peroxidase Assay Kit (Molecular
132 Probes, PoortGebouw, The Netherlands) based on the formation of an oxidation product
133 (resorufin) derived from the reaction between H_2O_2 given in excess and the Amplex Red
134 reagent. Cell lysates were used undiluted to perform the test. The absorbance was
135 determined with a Spectra Shell Microplate Reader using a 540 nm filter and read against
136 a standard curve of peroxidase ranging from 0.078 to 10 mU/ml.
137
138 *2.4.3 Catalase activity*
139 Catalase activity was measured by an Amplex Red Catalase assay kit (Molecular
140 Probes) based on formation of an oxidation product (resorufin) derived from the reaction
141 between H_2O_2 given in excess and the Amplex Red reagent in the presence of horseradish
142 peroxidase. Cell lysates were diluted 1:10 to perform the test and read against a standard
143 curve of catalase ranging from 62.5 to 1000 mU/ml. The absorbance was determined with
144 a spectra Shell Microplate reader using a 540 nm filter.
145
146 *2.5 Scavenging non-enzymatic activity.*
147 *2.5.1 Ferric reducing antioxidant power (FRAP) assay*
148 FRAP assay is a colorimetric method based on the ability of the antioxidant molecules
149 to reduce ferric-tripiridyltriazine (Fe^{3+}TPTZ) to a ferrous form (Fe^{2+}TPTZ) [25]. Fe^{2+} is
150 measured spectrophotometrically trough determination of its coloured complex with 2,4,6-
151 Tris(2- pyridyl)-s-triazine (Fe^{2+} TPTZ). TPTZ reagent was prepared before use, mixing 25 ml
152 of 300 mmol/l acetate buffer, 2.5ml of 10 mmol/l 2,4,6-Tris(2- pyridyl)-s-triazine (TPTZ)
153 in 40 mmol/l HCl and 2.5 ml of 20 mmol/l $FeCl_3 \cdot 6H_2O$ solution. Briefly, 2 $\times 10^5$ cells/200µl
154 CM were seeded in 96-well plates. The test was performed on 40µL of cell lysates obtained
155 as described above, added to Fe^{3+}TPTZ reagent and then incubated at 37°C for 30 min.
156 The absorbance of Fe^{2+} TPTZ was determined by Spectra Shell Microplate Reader at a

wavelength of 595nm. The ferric reducing ability of cell lysates was calculated by plotting a standard curve of absorbance against $FeSO^{4-}7H_2O$ standard solution.

2.6 Statistical analysis

Each experiment was repeated at least five times; for each cell culture, six replicates for each treatment were carried out. Data are presented as means ± SEM. Statistical analysis was performed by ANOVA using Statgraphics package (STC Inc., Rockville, MD, USA). When significant difference were found ($p < 0.05$), means were compared by Scheffè's F test.

3. Results

3.1. Granulosa cell proliferation

The BrdU incorporation assay showed no significant effect of STC1 treatment on granulosa cell proliferation (data not shown).

3.2. Granulosa cell redox status

The WST assay showed a increase ($p < 0.001$) in O_2^- production by granulosa cells treated with STC1. In particular, 5 ng/ml induced the highest increase ($p<0.001$) of the concentrations tested (Fig. 1).

H_2O_2 production was reduced ($p < 0.001$) by both STC1 concentrations without significant difference between them ($p < 0.001$) (Fig. 2).

3.3 Scavenging enzymatic activity

No significant changes in SOD activity were induced by the STC1 treatments (data not shown).

Peroxidase activity was inhibited ($p < 0.05$) by both STC1 treatments with no differences between the two doses tested ($p<0.05$) (Fig. 3).

183 Catalase activity was stimulated ($p < 0.01$) by the higher dose of STC1 while the lower
184 dose did not produce any significant effect (Fig. 4).

185 *3.4 Scavenging non-enzymatic activity*

186 The non-enzymatic antioxidant capacity of granulosa cells was stimulated ($p < 0.05$) by
187 STC1. In particular, the higher dose was the most effective ($p < 0.05$) (Fig. 5).

188

189

190 ## 4. Discussion

191 The mammalian form of STC1 was identified for the first time by Chang and co-workers
192 [26]. Since then, numerous studies have been undertaken to investigate the biological role
193 of this glycoprotein hormone. STC1 gene is expressed in different mammalian organs and, at
194 least in mice and man, the highest level is found in the ovary [7, 8]. Luo and coworkers [27]
195 found a high specific STC1 binding to ovarian granulosa cells in rats. Previous data from our
196 laboratory suggested that STC1 is expressed in swine follicles where the protein can
197 physiologically modulate steroidogenesis [18, 19]. SPOSTARE IN INTRO

198 First of all, we attempted to verify the potential modulatory role on granulosa cell
199 proliferation, an essential event for follicular development. In our experimental model, we
200 did not detect significant differences in cell proliferation due to STC1 treatments, as also
201 evidenced by Zlot and colleagues [28] in human endothelial cells.

202 The high metabolic activity of granulosa cells is a potential source of active free radicals
203 that can act as important second messengers [29]. ROS induce the expression of genes
204 involved in ovarian physiological processes such as oocyte maturation and follicular
205 development [30, 31].

206 In the present study, we show that STC1 affects the production of both O_2^- and H_2O_2 by
207 granulosa cells. In particular, a stimulatory effect of STC1 on O_2^- production has been
208 demonstrated. This finding could be related to the ability of STC1 to stimulate

209 mitochondrial activity in muscle, liver and kidney [32]. Since the mitochondria are the main
210 sites of O_2^- generation [33], the stimulatory effects of STC-1 on mitochondrial electron
211 transport chain could be responsible for the O_2^- increase. However, in cytokine-treated
212 endothelial cells, STC1 *reduces* O_2^- production [16]. Moreover, Wang and coworkers [34]
213 documented that STC1 inhibits O_2^- generation in macrophages through induction of
214 mitochondrial uncoupling protein UCP2. It has been hypothesized that STC1 activates anti-
215 oxidant pathways and plays a cytoprotective role in these cells [35]. *The reasons for these*
216 *discrepances could be explained by differences in cell types and experimental models.*

217 A series of reports document the involvement of O_2^- in modulating ovarian
218 steroidogenesis [30, 36, 37] and in inducing ovulation[30, 38]. Therefore, we can argue that
219 STC1 may influence these pivotal ovarian events by increasing O_2^- production.

220 Intracellular ROS levels are dependent on the balance between ROS production and
221 antioxidant activities [39]. The preovulatory follicle has powerful antioxidant defence
222 mechanism to counteract ROS generation [31]. Our results show that STC1 has no significant
223 effect on the activity of SOD, the enzyme that catalyses dismutation of O_2^- to H_2O_2. On the
224 contrary, STC1 is able to modulate both enzymes that perform H_2O_2 scavenging, catalase
225 and peroxidase. In particular, our results show that the higher STC1 concentration stimulates
226 catalase activity. Notably, this may explain STC1 inhibitory effect on H_2O_2 generation.
227 However, we also observed a H_2O_2 reduction at the lower STC1 concentration. Thus, STC1
228 can probably affect H_2O_2 levels by activating other pathways as demonstrated by the
229 ability of STC1 to increase the non-enzymatic scavenger activity in granulosa cells.

230 Moreover, our results reveal a STC1-induced decrease in peroxidase activity: this effect
231 might be triggered by the reduction in H_2O_2 generation since antioxidant scavenger
232 expression depends on ROS levels, which are their substrate [40]. Agarwal and coworkers
233 [31] documented that H_2O_2 inhibits the production of progesterone in human granulosa
234 cells and Gonzalez-Pacheco and coworkers [41] demonstrated that it induces the
235 expression of the main angiogenic factor, namely VEGF in endothelial cells. In our previous

236 work, [19] we have shown that STC1 increases progesterone production and inhibits VEGF
237 secretion [20] in swine granulosa cells. The present observations suggest that these effects
238 might be related to an STC1- induced H_2O_2 decrease.

239 In conclusion, the information obtained in the present study demonstrates that STC1
240 affects redox status of porcine granulosa cells. These data further support the role of STC1 as
241 a physiologic modulator of follicular function .

242

243

244 References

245 [1] Fenwick J, So Y. A perfusion study of the effect of stanniectomy on the net influx of
246 calcium-45 across an isolated eel gill. J Exp Zool 1974;188:125-31.

247 [2] Wagner GF, Milliken C, Friesen HG, Copp DH. Studies on the regulation and
248 characterization of plasma stanniocalcin in rainbow trout. Mol Cell Endocrinol
249 1991;79:129–38.

250 [3] Lu M, Wagner GF, Renfro JL. Stanniocalcin stimulates phosphate reabsorption by
251 flounder renal proximal tubule in primary culture. Am J Physiol 1994;267:1356–62.

252 [4] Wong CKC, Ho MA, Wagner GF. The co-localization of stanniocalcin protein, mRNA
253 and kidney cell markers in the rat kidney. J Endocrinol 1998; 158:183–9.

254 [5] Serlachius M, Zhang KZ, Andersson LC.. Stanniocalcin in terminally differentiated
255 mammalian cells. Peptides 2004;25:1657-62.

256 [6] Song G, Bazer FW, Wagner GF, Spencer TE. Stanniocalcin (STC) in the endometrial
257 glands of the ovine uterus: regulation by progesterone and placental hormones. Biol
258 Reprod 2006;74:913-22.

259 [7] Varghese R, Wong CKC, Deol H, Wagner GF, DiMattia GE. Comparative analysis of
260 mammalian stanniocalcin genes. Endocrinology 1998;139:4714-25.

[8] Deol HK, Varghese R, Wagner GF, Dimattia GE. Dynamic regulation of mouse ovarian stanniocalcin expression during gestation and lactation. Endocrinology 2000;141:3412-21.

[9] Olsen HS, Cepeda MA, Zhang QQ, Rosen CA, Vozzolo BL. Human stanniocalcin: a possible hormonal regulator of mineral metabolism. Proc Natl Acad Sci 1996;93:1792-6.

[10] Madsen KL, Tavernini MM, Yachimec C, Mendrick DL, Alfonso PJ, Buergin M, Olsen HS, Antonaccio MJ, Thomson AB, Fedorak RN. Stanniocalcin: a novel protein regulating calcium and phosphate transport across mammalian intestine. Am J Physiol 1998;274:G96-G102.

[11] Li K, Dong D, Yao L, Dai D, Gu X, Guo L.. Identification of STC1 as an -amyloid activated gene in human brain microvascular endothelial cells using cDNA microarray. Biochem Biophys Res Commun 2008;376:399-403.

[12] Block GJ, Ohkouchi S, Fung F, Frenkel J, Gregory C, Pochampally R, DiMattia G, Sullivan DE, Prockop DJ. Multipotent Stromal Cells Are Activated to Reduce Apoptosis in Part by Upregulation and Secretion of Stanniocalcin-1. Stem Cell 2009;7:670-81.

[13] Huang L, Garcia G, Lou Y, Zhou Q, Truong LD, DiMattia G, Lan XR, Lan HY, Wang Y, Sheikh-Hamad D. Anti-inflammatory and renal protective actions of stanniocalcin-1 in a model of anti-glomerular basement membrane glomerulonephritis. Am J Pathol 2009;174:1368-78.

[14] Song G, Dunlap KA, Kim J, Bailey DW, Spencer TE, Burghardt RC, Wagner GF, Johnson GA, Bazer FW: Stanniocalcin 1 is a luminal epithelial marker for implantation in pigs regulated by progesterone and estradiol. Endocrinology 2009;150:936-45.

[15] Chakraborty A, Elbjeirami W, Sheikh-Hamad D, McReynolds MR, Hoying JB, Bick R, Truong L, Poindexter B, Hui L, Brooks H, Zhang P, Smith W. Stanniocalcin-1 regulates

287 endothelial gene expression and modulates transendothelial migration of leukocytes.
288 Am J Physiol Renal Physiol 2007;292:895-904.

289 [16] Chen C, Jamaluddin S, Yan S, Sheikh-Hamad D, Yao Q. Human Stanniocalcin-1
290 Blocks TNF-alpha–Induced Monolayer Permeability in Human Coronary Artery
291 Endothelial Cells. Arterioscler Thromb Vasc Biol 2008;28:906-12.

292 [17] Holmes DIR, Zachary IC. Vascular endothelial growth factor regulates Stanniocalcin-1
293 expression via Neuropilin-1-dependent regulation of KDR and synergism with
294 fibroblast growth Factor-2. Cell Signal 2008;20:569-79.

295 [18] Basini, G., Baioni, L., Bussolati, S., Grolli, S., Kramer, L., Wagner, G.F., Grasselli, F., 2010.
296 Expression and localization of stanniocalcin 1 in swine ovary. General and
297 Comparative Endocrinology. 166, 404-408.

298 [19] Baioni L, Basini G, Bussolati S, Grasselli F. Stanniocalcin 1 is a potential physiological
299 modulator of steroidogenesis in the swine ovarian follicle. Vet Res Commun
300 2009;33:73-6.

301 [20] Basini G, Bussolati S, Santini SE, Grasselli F. Stanniocalcin, a potential ovarian
302 angiogenesis regulator, does not affect endothelial cell apoptosis. Ann NY Acad Sci
303 2009;1171:94-9.

304 [21] Basini G, Bianco F, Grasselli F, Tirelli M, Bussolati S, Tamanini C. The effects of reduced
305 oxygen tension on swine granulosa cell. Regul Pept 2004;120:69-75.

306 [22] Basini G, Grasselli F, Bianco F, Tirelli M, Tamanini C. Effect of reduced oxygen tension
307 on reactive oxygen species production and activity of antioxidant enzymes in swine
308 granulosa cells. Biofactors 2004;20:61-9.

309 [23] Agarwal A, Gupta S, Sikka S. The role of free radicals and antioxidants in
310 reproduction. Curr Opin Obstet Ginecol 2006;18:325-332.

311 [24] Basini G, Tringali C, Baioni L, Bussolati S, Spatafora C, Grasselli F. Biological effects on
312 granulosa cells of hydroxylated and methylated resveratrol analogues. Mol Nutr Food
313 Res 2010;54:S236-43.
314 [25] Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of
315 "antioxidant power": the FRAP assay. Anal Biochem 1996;239:70-6.
316 [26] Chang AC, Janosi J, Hulsbeek M, de Jong D, Jefrey KJ; Noble JR, Reddel RR. A novel
317 human cDNA higlhly homologous to the fish hormone stanniocalcin. Mol Cell
318 Endocrinol 1995;112:241-7.
319 [27] Luo CW, Kawamura K, Klein C, Hsueh AJ. Paracrine regulation of ovarian granulosa
320 cell differentiation by stanniocalcin (STC) 1: mediation through specific STC1 receptors.
321 Mol Endocrinol 2004;18:2085-96.
322 [28] Zlot C, Ingle G, Hongo J, Yang S, Sheng Z, Schwall R, Paoni N, Wang F, Peale FV,
323 Gerritsen ME. Stanniocalcin 1 is an autocrine modulator of endothelial angiogenic
324 responses to hepatocyte growth factor. J Biol Chem 2003;278:47654-9.
325 [29] Basini G, Bussolati S, Santini SE, Grasselli F. Reactive oxygen species and anti-oxidant
326 defences in swine follicular fluids. Reprod Fertil Dev 2008;20:269-74.
327 [30] Berhman HR, Kodaman PH, Preston SL, Gao S. Oxidative stress and the ovary. J Soc
328 Gynecol Investig 2001;8:40-2.
329 [31] Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction.
330 Reprod Biol Endocrinol 2005;14: 3-28.
331 [32] Ellard JP, McCudden CR, Tanega C, James KA, Ratkovic S, Staples JF, Wagner GF. The
332 respiratory effects of stanniocalcin-1 (STC-1) on intact mitochondria and cells: STC-1
333 uncouples oxidative phosphorylation and its actions are modulated by nucleotide
334 triphosphates. Mol Cell Endocrinol 2007;264:90–101.
335 [33] Murphy MP. How mitochondria produce reactive oxygen species. Biochem J
336 2009;417:1-13.

337 [34] Wang Y, Huang L, Abdelrahim M, Cai Q, Truong A, Bick R, Poindexter, B., Sheikh-
338 Hamad, D., 2009. Stanniocalcin-1 suppresses superoxide generation in macrophages
339 through induction of mitochondrial UCP2. J. Leuckoc Biol. 86, 981-988.
340 [35] Sheik Hamad D. Mammalian stanniocalcin-1 activates mitochondrial anti-oxidant
341 pathways: new paradigms for the regulation of macrophages and endothelium. Am J
342 Renal Physiol 2010;298:248-54.
343 [36] Sawada M, Carlson JC. Intracellular regulation of progesterone secretion by the
344 superoxide radical in the rat corpus luteum. Endocrinology 1996;137:1580-4.
345 [37] Jain S, Saxena D, Kumar PG, Laloraya M. NADPH dependent superoxide generation
346 in the ovary and uterus of mice during estrous cycle and early pregnancy. Life Sci
347 2000;66:1139-46.
348 [38] Fujii J, Iuchi Y, Okada F. Fundamental roles of reactive oxygen species and protective
349 mechanisms in the female reproductive system. Reprod Biol Endocrinol 2005;3:43-53.
350 [39] Frey RS, Ushio-Fukai M, Malik AB. NADPH Oxidase-Dependent Signaling in
351 Endothelial Cells: Role in Physiology and Pathophysiology. Antioxid Redox. Signal
352 2009;11:791-810.
353 [40] Scandalios JC. Oxidative stress: molecular perception and transduction of signals
354 triggering antioxidant gene defenses. Braz J Med Biol Res 2005;38:995-1014.
355 [41] González-Pacheco FR, Deudero JJP, Castellanos MC, Castilla MA, A´lvarez-Arroyo
356 MV, Yague S, Caramelo C. Mechanisms of endothelial response to oxidative
357 aggression: protective role of autologous VEGF and induction of VEGFR2 by H_2O_2.
358 Am J Physiol Heart Circ Physiol 2006;291:1395–1401.

ALLEGATO D

"The axonal guidance factor netrin-1 as a potential modulator of swine follicular function"

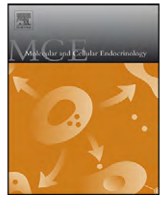
Molecular and Cellular Endocrinology. 2010. 331: 41-48. doi: 10.1016/j.mce.2010.08.001

Molecular and Cellular Endocrinology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce



The axonal guidance factor netrin-1 as a potential modulator of swine follicular function

G. Basini[a,*], C. Cortimiglia[a], L. Baioni[a], S. Bussolati[a], S. Grolli[b], R. Ramoni[b], F. Grasselli[a]

[a] Sezione di Fisiologia Veterinaria, Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti, Via del Taglio 10, Università degli Studi di Parma, 43126 Parma, Italy

[b] Sezione di Biochimica Veterinaria, Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti, Via del Taglio 10, Università degli Studi di Parma, 43126 Parma, Italy

ARTICLE INFO

Article history:
Received 31 March 2010
Received in revised form 2 August 2010
Accepted 2 August 2010

Keywords:
Ovary
Angiogenesis
Steriods

ABSTRACT

This study was aimed to improve knowledge about swine ovarian follicular function, paying attention to angiogenesis, since new vessel growth is a fundamental event in ovarian function. In particular, we investigated a potential involvement of netrin-1, a protein known as a guidance axon factor.

Firstly, we studied the expression and immunolocalization of netrin-1 in swine ovarian follicle and its effect on cultured swine granulosa cell viability and steroidogenesis. Furthermore, aortic endothelial cells were employed to verify a possible netrin-1 effect on angiogenesis.

Our data demonstrate the expression and the presence of netrin-1 in swine follicular fluid; in addition, it was shown that netrin-1 inhibits granulosa cell viability and estradiol 17β levels while it stimulates progesterone production. Netrin-1 also inhibits aortic endothelial cell growth in the angiogenesis bioassay. This effect appears to be mediated by inhibiting vascular endothelial growth factor and stimulating nitric oxide.

Therefore, we hypothesize that netrin-1 could be important for follicular function in the swine.
© 2010 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Netrins are a family of highly conserved secreted proteins structurally related to laminins. They were named after the Sanskrit word “netr”, which means “one who guides”, since they possess guidance cues in axon pathfinding. Recent studies report the involvement of netrins in other developmental process, including the formation of vessels (Cirulli and Yebra, 2007). Both neuronal and vascular development require guidance to establish a precise branching pattern and it has been shown that several molecules implicated in axonal navigation have a similar role in regulating vessel sprouting (Larrivée et al., 2009). In particular, netrin-1 has been related to the fine-tuning of the angiogenic process and published data demonstrate pro- and anti-angiogenic effects (Park et al., 2004; Larrivée et al., 2007; Bouvrée et al., 2008; Castets et al., 2009). Maeda et al. (2008) recently demonstrated that netrin-1 is expressed in swine granulosa cells suggesting that the protein could be involved in the modulation of ovarian follicle function. In particular, netrin-1 could play a role in the regulation of the marked cyclical angiogenesis which takes place in the developing follicle and represents an unvaluable physiological model for the investigation of the molecular events responsible for new vessel growth.

On these bases, the present study was undertaken to explore the hypothesis that netrin-1 could act as a physiological modulator of swine follicular function.

The expression and the immunolocalization of netrin-1 in swine ovarian follicle were explored and the effect of different protein concentrations on the main parameters of swine granulosa cell function were examined. Moreover, an angiogenesis bioassay set up in our laboratory (Basini et al., 2008a) was employed to evaluate the effect of netrin-1 on new vessel growth.

2. Materials and methods

All reagents used in this study were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

2.1. Collection of ovaries

Swine ovaries were collected at a local slaughterhouse from Large White crossbred gilts, placed into cold PBS (4 °C) supplemented with penicillin (500 IU/ml), streptomycin (500 μg/ml) and amphotericin B (3.75 μg/ml), maintained in a freezer bag and transported to the laboratory within 1 h.

2.2. Netrin-1 in swine ovarian follicle

2.2.1. Immunohistochemistry

Ovaries were placed in 10% buffered formalin and paraffin embedded. Following routine processing, 6 μm-thick sections were placed on polylysine-treated slides

* Corresponding author.
E-mail address: basini@unipr.it (G. Basini).

0303-7207/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2010.08.001

and stained with a polyclonal antiserum raised against a peptide corresponding to 501–604 amino acid sequence of human netrin-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, sections were blocked in 10% normal swine serum for 20 min (200 μl) and then the primary antibody was applied (250 μl of a solution diluted 1:100 in PBS) and incubated overnight at 4 °C. After rinsing in PBS, the slides were incubated for 15 min with 200 μl of a biotinylated secondary antibody. After three 5-min washes in PBS, 200 μl of a solution of streptavidin–peroxidase complex (LSAB®+SYSTEM-HRP, Dako, Glostrup, Denmark) was applied for 15 min. Positive reactions were revealed by aminoethylcarbazole (AEC) (Dako, Glostrup, Denmark). Sections were washed 3 times for 5 min with PBS and counterstained with Gill's ematoxylin for 2 min. Control sections were subjected to the same procedure, except that diluted swine serum replaced the primary antibody.

2.2.2. *Western blotting of follicular fluid protein content*

Follicular fluid was collected with a 26-gauge from follicles >5 mm (Basini et al., 2008b), centrifuged (300 × *g*; 10 min) to separate rapidly the fluid from the cell fraction and refrigerated at −80 °C until assayed. As a positive control, a sample of soluble protein extract from porcine spinal marrow was also prepared. To this aim few grams of tissue were collected from a freshly slaughtered pig and homogenized by Ultra-Turrax (one stroke of 30 s at maximum speed) in 20 mM Tris/HCl pH 7.8 (1 ml of buffer/gram of tissue). The suspension was centrifuged (18,000 × *g*) for 5 min and the supernatant was stored −80 °C.

The protein pattern of follicular fluid and spinal marrow samples were resolved by 10% sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). After electrophoresis, the protein bands were transferred onto a ProBlott membrane (Applied Biosystem, Carlsbad, CA) (Towbin et al., 1979) that was blocked with 5% skimmed dry milk dissolved in 20 mm Tris/HCl buffer, pH 7.8 containing 150 mm NaCl, and 0.01% (w/v) Tween 20 (TTBS). After washing with TTBS the membrane was incubated overnight with an anti-netrin-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) raised in rabbit diluted (1:50) in TTBS containing 1% (w/v) skimmed dry milk. After three washes in TTBS the membrane was incubated 45 min with a solution of a biotinylated anti-rabbit IGg antibody (LSAB®+SYSTEM-HRP, Dako, Glostrup, Denmark) diluted 1:5 in TTBS. A solution of complex of streptavidin–peroxidase was then applied for 15 min and positive reactions were revealed by AEC. A replicate SDS-PAGE gel was stained with Comassie blue.

2.2.3. *RNA extraction and RT-PCR*

Follicles >5 mm were dissected from the ovaries according to Basini et al. (2010). Each follicle was cut open, granulosa cells were separated by means of using a siliconized Pasteur pipette and centrifuged at 300 × *g* for 10 min. Theca tissue was peeled from each follicle using a forceps, deep frozen in liquid nitrogen and powdered in a mortar.

Total RNA was extracted using Nucleospin® RNA II (Macherey-Nagel Gmbh, Duren, Germany) according to the manufacturer's instructions. Total RNA was quantified by absorbance at 260 nm (Gen Quant Pro, Amersham Bioscences, Freiburg, Germany). Total RNA (2 μg) was reverse transcribed with Ready-to-Go You Prime First-Strands Beads (Amersham Biosciences, Freiburg, Germany). Netrin-1 cDNA was PCR amplified in parallel with β actin cDNA as an internal positive control. For netrin-1, the sense primer (5′-GAG ACC TTC AAC ACG CCG-3′) and antisense primer (5′-GGA AGG TGG ACA GCG AGG-3′) (MWG Biotec, Ebersberg, Germany) were derived from Gene Bank sequence of porcine netrin-1 (accession number DQ368597). Swine actin was amplified using the primers pACTIN sense (5′-GAG ACC TTC AAC ACG CCG-3′) and pACTIN antisense (5′-GGA AGG TGG ACA GCG AGG-3′) (MWG Biotec, Ebersberg, Germany). An aliquot (5 μl) of the cDNA template was amplified by PCR using 0.5 μl (500 mU) Taq polymerase (Fermentas, Hannover, MD, USA) in 50 μl PCR buffer containing 10 mM dNTP mix (Fermentas), 25 μM for netrin-1 primers and 5 μM for Actin primers. Amplification was carried out using the thermal cycler PTC-100 Peltier (MJ Research, San Francisco, CA, USA). After an initial denaturation step for 5 min at 95 °C, target cDNA was amplified for 3 cycles with a denaturation step at 95 °C for 30 s, annealing at 60 °C for 30 s (−1 °C in the second and third cycle), elongation at 72 °C for 30 s, and for 35 cycles with a denaturation step at 95 °C for 30 s, annealing at 57 °C for 30 s, elongation at 72 °C for 30 s. Reactions were terminated with a final elongation at 72 °C for 10 min. The PCR products were separated on 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml) and visualized under UV light. Digital images were captured by Power Shot A610 photo camera (Canon, Tokyo, Japan). Quantification of band intensity was performed with NIH Image software (Scion Corporation, MA, USA, http://rsb.info.nih.gov/nih-image/).

2.3. *Netrin-1 effects on swine granulosa cell functions*

2.3.1. *Granulosa cell culture*

Granulosa cells were aseptically harvested by aspiration of follicles with a 26-gauge needle, released in medium containing heparin (50 IU/ml), centrifuged for pelleting and then treated with 0.9% prewarmed ammonium chloride at 37 °C for 1 min to remove red blood cells. Cell number and viability were estimated using a haemocytometer under a phase contrast microscope after vital staining with trypan blue (0.4%) of an aliquot of the cell suspension. Cells were seeded in DMEM/Ham's F12 supplemented with sodium bicarbonate (2.2 mg/ml), bovine serum albumin (BSA 0.1%), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), selenium (5 ng/ml) and transferrin (5 μg/ml). Once seeded, cells were incubated for 48 h at 37 °C under humidified atmosphere (5% CO_2) in the presence or absence of netrin-1 at the concentration of 5, 30 and 100 ng/ml. The protein was produced as reported by Serafini et al. (1994). Briefly, cDNAs encoding chick netrin-1 tagged with a c-myc epitope at its C-terminus and fused to the constant (Fc) region of the human IgG1, were subcloned into the expression vector pCEP4 (Invitrogen, Carlsbad, CA) and used to transfect 293-EBNA cells (Invitrogen, Carlsbad, CA). Protein was finally purified from conditioned media by heparin affinity chromatography to 85–90% homogeneity, as assessed by silver staining (Keino-Masu et al., 1996).

2.3.2. *Granulosa cell viability*

2×10^5 cells were seeded in 96-well plates in 200 μl CM. Cell proliferation was assayed using a bioluminescent assay (ATP-lites; Packard Bioscience, Groningen, Netherlands) which measured intracellular ATP levels. ATP, being present in all metabolically active cells, is a viability marker whose concentration declines very rapidly when the cells undergo necrosis or apoptosis. The ATP lite-M assay system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. The emitted light is proportional to the ATP concentration. Briefly, 50 μl of mammalian cell lysis solution were added to 100 μl of cell suspension and the plate was shaken for 5 min in an orbital shaker at 700 rpm in order to lyse the cells and stabilize ATP. Then 50 μl of substrate solution were added to the wells and the microplate was shaken for 5 min in an orbital shaker at 700 rpm. The plate was placed in the dark for 10 min and the luminescence was measured in a luminometer (Victor, Packard Bioscience, Groningen, Netherlands).

2.3.3. *Granulosa cell steroid production*

10^4 cells/well were seeded in 96-well plates in 200 μl of CM supplemented with androstenedione (28 ng/ml). Culture media were then collected, frozen and stored at −20 °C until progesterone (P4) and estradiol 17β (E2) were determined by a validated Radio Immuno Assays (Grasselli et al., 1993). P4 assay sensitivity and ED_{50} were 0.24 and 1 nmol/l, respectively; E2 assay sensitivity and ED_{50} were 0.05 and 0.2 nmol/l. The intra- and inter-assay coefficients of variation were less than 12% for both assays.

2.4. *Netrin-1 effects on swine aortic endothelial cell functions*

2.4.1. *Aortic endothelial cell culture*

An immortalized porcine aortic endothelial cell line (AOC) (Carrello et al., 2002) was generously provided by José Yelamos (Hospital Universitario Virgen de la Arrixaca, El Palmar, 30120 Murcia, Spain). Cells were cultured in M199 medium supplemented with sodium bicarbonate (2.2 mg/ml), FCS 20%, penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml). In all experiments, AOC at 19th passage were used.

2.4.2. *Angiogenesis bioassay*

The microcarrier-based fibrin gel angiogenesis assay was performed as described by Basini et al. (2008a) with some modifications. Briefly, 12.5 mg gelatin-coated cytodex-3 microcarriers in 1 ml PBS were incubated for 3 h to hydrate. After two washings in PBS and one in CM, the microcarriers were put in flasks containing 5 ml CM; AOC (5×10^5) were added and cultured for 24 h in order to let the endothelial cells coat the microcarriers. For the fibrin gel preparation, 40 μl microcarriers covered by AOC were pipetted into 6 well plates containing a solution of fibrinogen (1 mg/ml PBS, pH 7.6), added with 1250 IU thrombin (250 μl). Fibrin gels were allowed to polymerize for 30 min at 37 °C, and then were equilibrated for 60 min with 2 ml M199. After a change of the medium, AOC were incubated in the presence or absence of 5, 30 or 100 ng/ml of netrin-1. Plates were incubated at 37 °C under humidified atmosphere (5% CO_2). AOC were cultured for 96 h, renewing totally the treatment after 48 h as described above.

2.4.3. *Quantification of AOC growth on fibrin gel matrix*

Endothelial cell proliferation in the fibrin gel matrix was evaluated by means of the public domain NIH Program Scion Image Beta 4.02. Ten pictures were taken for each gel at 48 and 96 h; images were converted into gray scale, resized to 50% (Paintbrush Software, MS Office) and saved as Bitmap 24bit format compatible with Scion. The modified images were then imported into the program and measurements were made drawing the perimeter of the area occupied by AOC expressed as number of pixel. In order to validate the measurement of the area covered by AOC in fibrin gels as a reliable method to evaluate cell proliferation, fibrin gels were stained by the nuclear dye bis-benzimide (Hoechst 33258, 20 μg/ml in PBS for 60 min) and examined by the fluorescence microscope. This procedure was performed 20 times; for each experiment the number of nuclei was counted under fluorescence and pictures of the area covered by AOC were taken in order to measure the surface covered in the fibrin gel. A strong correlation was observed between the area covered by AOC and the number of nuclei found in the same area ($r = 0.96$).

2.4.4. *Aortic endothelial cell VEGF production*

2×10^5 cells were seeded in 96-well plates in 200 μl M199, in the presence or absence of 5, 30 or 100 ng/ml of netrin-1 and incubated for 48 h. VEGF content in culture media was quantified by an ELISA (Quantikine, R&D System, Minneapolis, MI, USA); this assay, developed for human VEGF detection, has been validated for

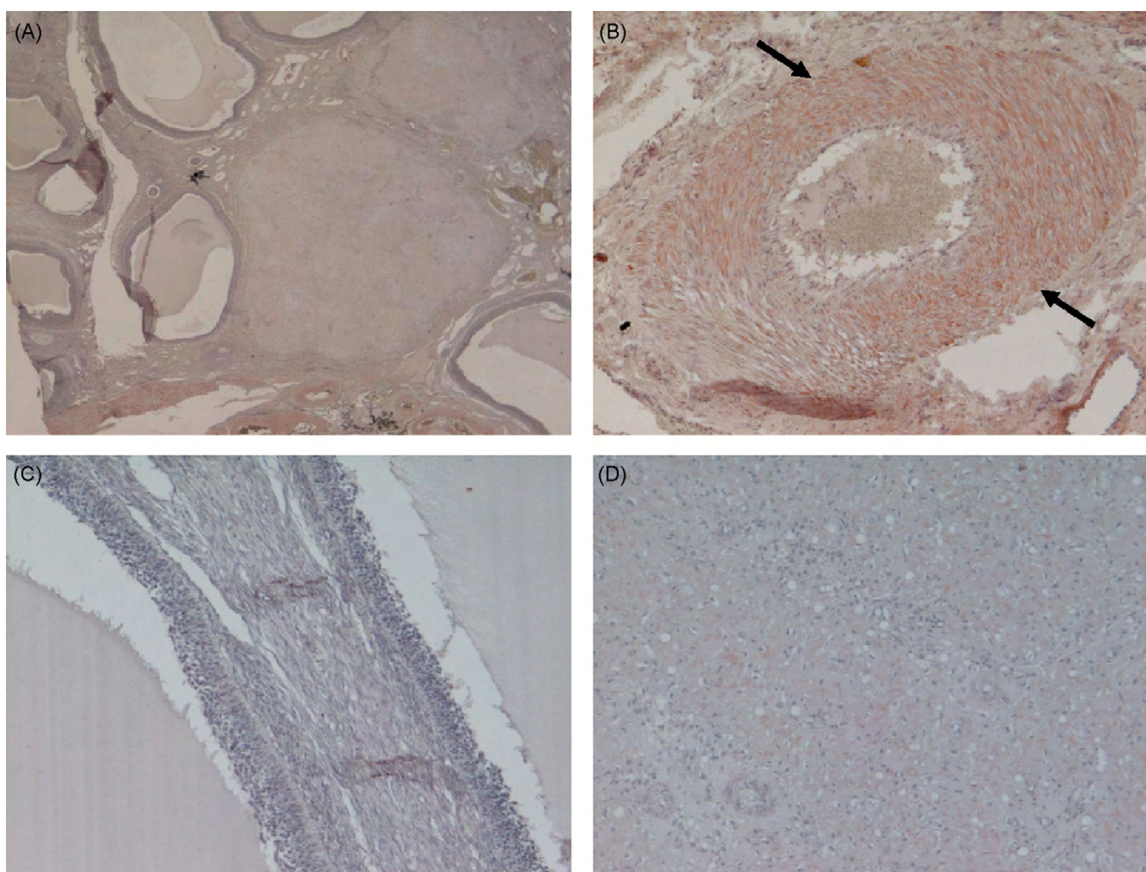


Fig. 1. Immunohistochemical localization of Netrin 1 in swine ovary. (A) Antral follicles, (B) vessel, (C) follicle walls, and (D) corpus luteum. A positive signal has been detected only in vessels (B, see arrows).

pig VEGF (Barboni et al., 2000). The assay sensitivity was 8.74 pg/ml, the inter- and intra-assay CVs were always less than 7%. Victor Reader set to read at a wavelength of 450 nm emission was used to quantify the reaction product.

2.4.5. Aortic endothelial cell NO production

2×10^5 cells were seeded in 96-well plates in 200 μl M199, in the presence or absence of 5, 30 or 100 ng/ml of netrin-1 and incubated for 48 h. Nitric oxide (NO) was assessed by measuring nitrite levels in culture media by microplate method based on the formation of a chromophore after reaction with Greiss reagent, which was prepared fresh daily by mixing equal volumes of stock A (1% sulfanilamide, 5% phosphoric acid) and stock B (0.1% N-[naphtyl] ethylenediamine dihydrochloride). After incubation with Greiss reagent the absorbance was determined with the Victor Reader using a 540 nm against 620 nm filter. A calibration curve ranging from 25 to 0.39 μM was prepared by diluting sodium nitrite in culture medium.

2.5. Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed by means of ANOVA using Statgraphics package (STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by Scheffè's *F*-test. *p*-Values <0.05 were considered to be statistically significant.

3. Results

3.1. Netrin-1 in swine ovarian follicle

3.1.1. Immunohistochemistry

Immunostaining of netrin-1 in swine ovarian follicle wall was undetectable. On the contrary, immunoreaction was localized in the ovarian vessels (Fig. 1B).

3.1.2. Follicular fluid Western blotting

The immunoblotting showed the presence of netrin-1 in swine follicular fluid (Fig. 2). The band corresponding to netrin-1, in fact, was resolved at the expected molecular weight of about 70 kDa both in follicular fluid and in a positive control consisting of a soluble protein extract from porcine spinal marrow.

3.1.3. Netrin-1 gene expression

Netrin-1 expression was evidenced both in granulosa and theca layer from swine follicles (Fig. 3).

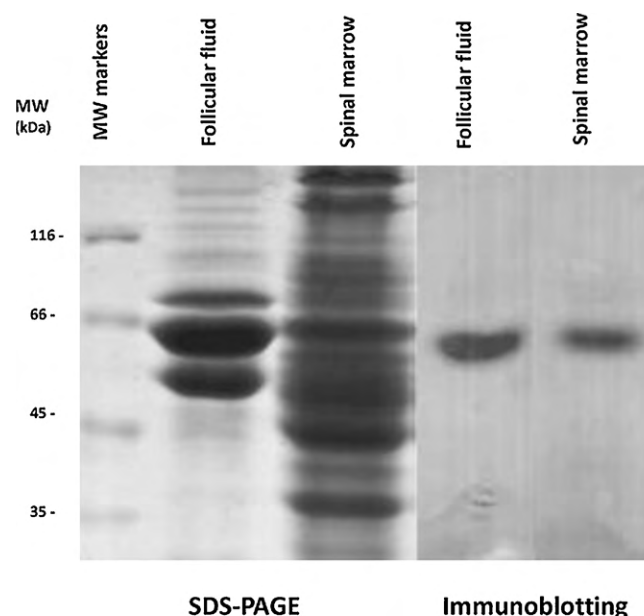


Fig. 2. Netrin 1 protein in swine follicular fluid and spinal marrow revealed by immunoblotting. Protein bands on SDS-PAGE gel were stained with Comassie blue.

Please cite this article in press as: Basini, G., et al., The axonal guidance factor netrin-1 as a potential modulator of swine follicular function. Mol. Cell. Endocrinol. (2010), doi:10.1016/j.mce.2010.08.001

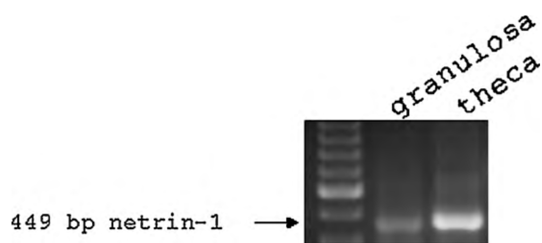


Fig. 3. Netrin-1 expression in swine granulosa and theca cells collected from swine follicles. The representative ethidium bromide-stained gel shows ACT and STC 1 amplicons from at least three follicles.

3.2. Netrin-1 effects on swine granulosa cell functions

3.2.1. Granulosa cell proliferation

Granulosa cell viability, evaluated as ATP content, was slightly but significantly ($p < 0.05$) inhibited by the treatment with 30 and 100 ng/ml of netrin-1 for 48 h. The lowest concentration of netrin-1 tested, 5 ng/ml, was ineffective (Fig. 4).

3.2.2. Granulosa cell steroid production

Granulosa cell steroid production, evaluated by RIA assays, was affected by netrin-1 treatment for 48 h. E2 production was significantly inhibited ($p < 0.01$) by 30 and 100 ng/ml of netrin-1 (Fig. 5); the same concentrations, on the contrary, stimulated ($p < 0.001$) P4 production (Fig. 6). The lowest concentration tested, 5 ng/ml, was ineffective on both steroid secretions (Figs. 5 and 6).

3.3. Netrin-1 effects on swine aortic endothelial cell functions

3.3.1. Angiogenesis bioassay

Netrin-1 significantly inhibited ($p < 0.05$) new vessel growth both after 48 and 96 h of incubation. All the concentrations tested (5, 30 and 100 ng/ml) exerted the same effect. (Figs. 7 and 8).

3.3.2. Aortic endothelial cell VEGF production

VEGF secretion by AOC was significantly inhibited ($p < 0.01$) by the 48 h treatment with all the examined concentrations of netrin-1 (5, 30 and 100 ng/ml) (Fig. 9).

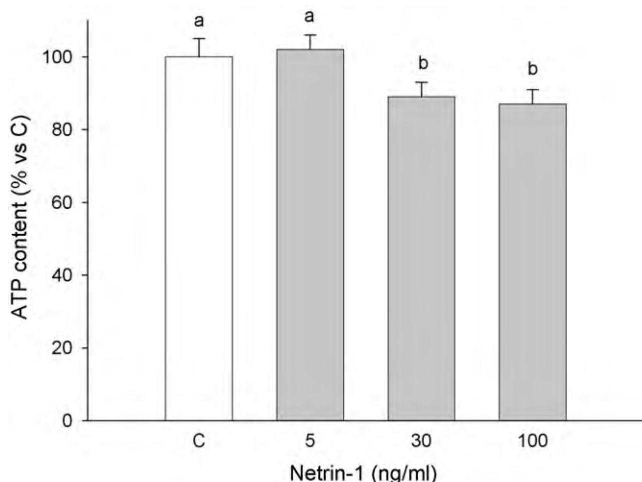


Fig. 4. Effect of the 48 h treatment with or without (C) netrin-1 at the concentrations of 5, 30 or 100 ng/ml on granulosa cell viability evaluated as ATP content. Data, expressed as % vs respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.05$).

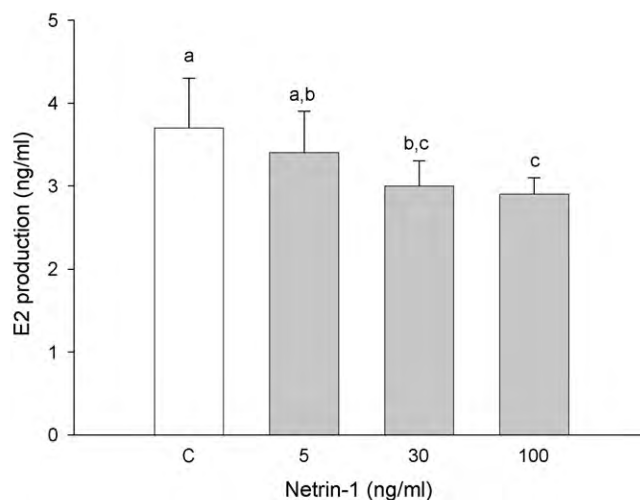


Fig. 5. Effect of the 48 h treatment with or without (C) netrin-1 at the concentrations of 5, 30 or 100 ng/ml on E2 production in swine granulosa cell culture evaluated by RIA. Data, expressed as ng/ml, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. The absence of common letters indicates a significant difference ($p < 0.01$).

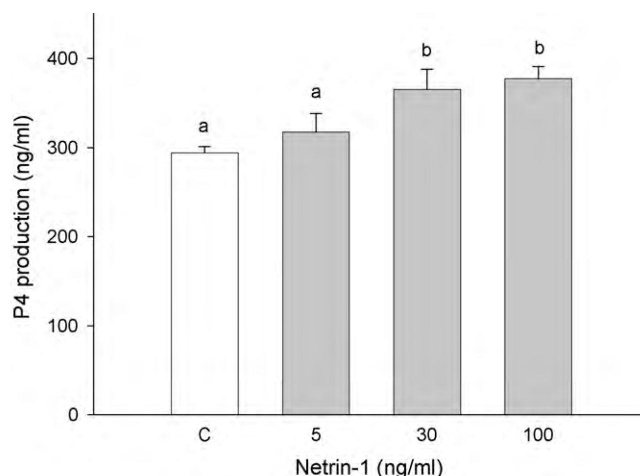


Fig. 6. Effect of the 48 h treatment with or without (C) netrin-1 at the concentrations of 5, 30 or 100 ng/ml on P4 production in swine granulosa cell culture evaluated by RIA. Data, expressed as ng/ml, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.001$).

3.3.3. Aortic endothelial cell NO production

All netrin-1 concentrations (5, 30 and 100 ng/ml) significantly stimulated ($p < 0.01$) NO generation after 48 h of incubation (Fig. 10).

4. Discussion

Ovarian physiology is connoted by follicular growth, a fundamental event which is strictly dependent on cell proliferation, steroid production and new vessel growth. The molecular machinery which regulates ovarian angiogenesis, though extensively studied, is far to be completely clarified. In a recent review, Larrivée et al. (2009) observe that anatomical and structural similarities between blood vessels and nerves have been highlighted by several studies. Among the molecules with attractive and repulsive properties which have been found to modulate the proper guidance of both nerves and blood vessels, netrin-1 displays both pro- and anti-angiogenic effects (Park et al., 2004; Castets et al., 2009).

Please cite this article in press as: Basini, G., et al., The axonal guidance factor netrin-1 as a potential modulator of swine follicular function. Mol. Cell. Endocrinol. (2010), doi:10.1016/j.mce.2010.08.001

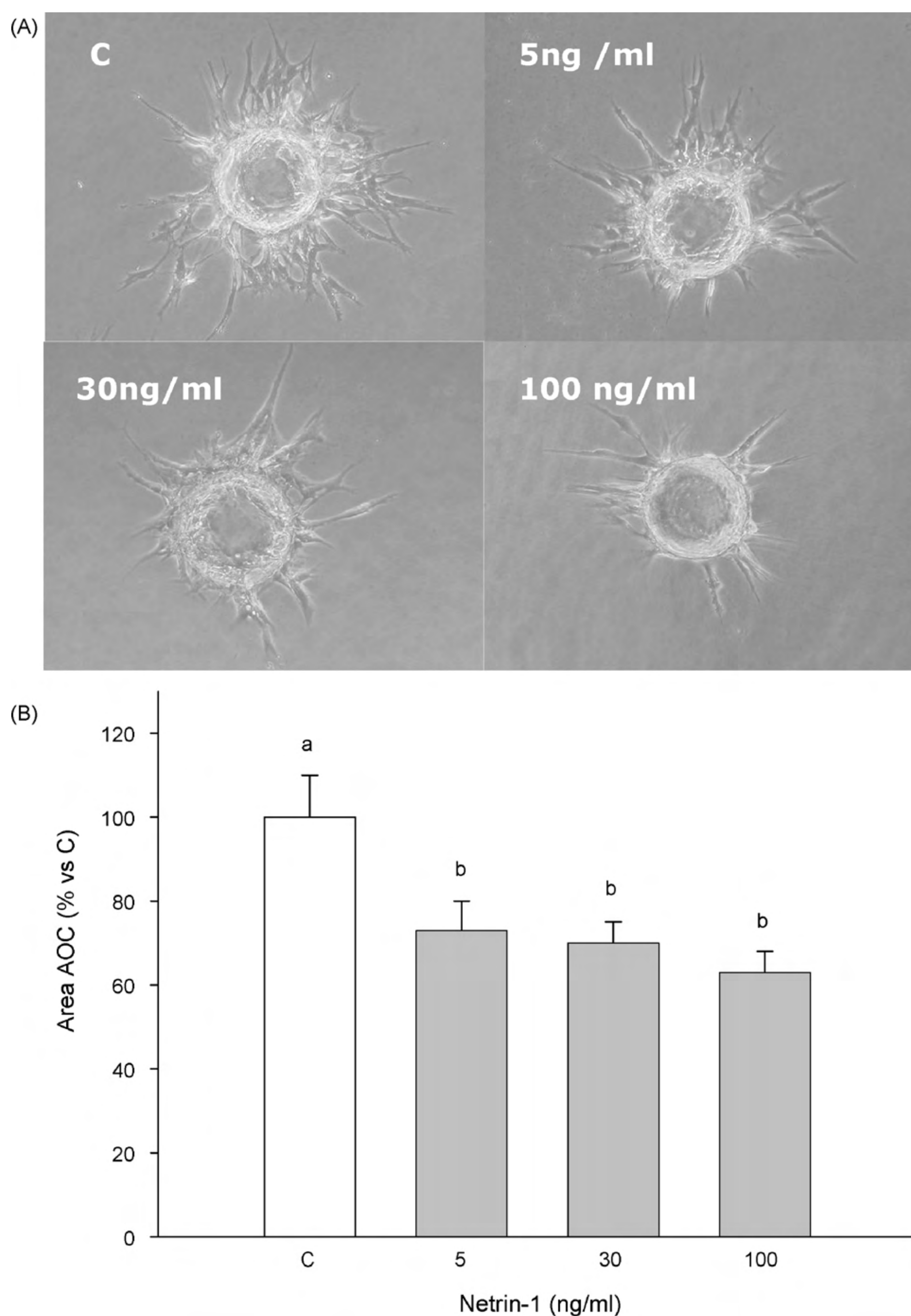


Fig. 7. Effect of the 48 h treatment with or without (C) netrin-1 at the concentrations of 5, 30 or 100 ng/ml on AOC growth in fibrin gel. Panel (A) phase contrast micrographs showing AOC growth at 48 h in fibrin gel matrix. Panel (B) area covered by AOC. Data, expressed as % vs respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.05$).

Interestingly, netrin-1 has been found to be expressed in swine granulosa cells from 3-5 mm ovarian follicles, thus suggesting its potential function in follicular function regulation (Maeda et al., 2008).

In the present study netrin-1 was evidenced in ovarian vessels but was undetectable in follicular wall. In addition, we document its expression both in granulosa and the theca layer from swine ovarian follicles >5 mm as well as in swine follicular fluid. These data lead us to hypothesize that netrin-1 could play a regulatory role in the function of the ovarian follicle, a structure which represents a typical example of events of proliferation and differentiation. Similarly, Strizzi et al. (2008) demonstrated that mammary gland, a structure subjected to different stages of development and differentiation, expresses netrin-1, which appears to be involved in the fine-tuning of these complex events. In particular, our data show that the protein inhibits granulosa cell viability and E2 synthesis, while it increases P4 production, thus suggesting its involvement in the induction of granulosa cell terminal differentiation. The relationship between growth and differentiation has been a matter of great interest in cellular biology. In granulosa cells, the differentiative function and growth-related process appear inversely controlled (Basini et al., 1998): netrin-1 could play a pivotal role in the paracrine/autocrine regulation of these events.

Since follicular growth is strictly dependent on adequate vascular development, this structure has been suggested as an invaluable physiological model of angiogenesis, a process which represents an

Please cite this article in press as: Basini, G., et al., The axonal guidance factor netrin-1 as a potential modulator of swine follicular function. Mol. Cell. Endocrinol. (2010), doi:10.1016/j.mce.2010.08.001

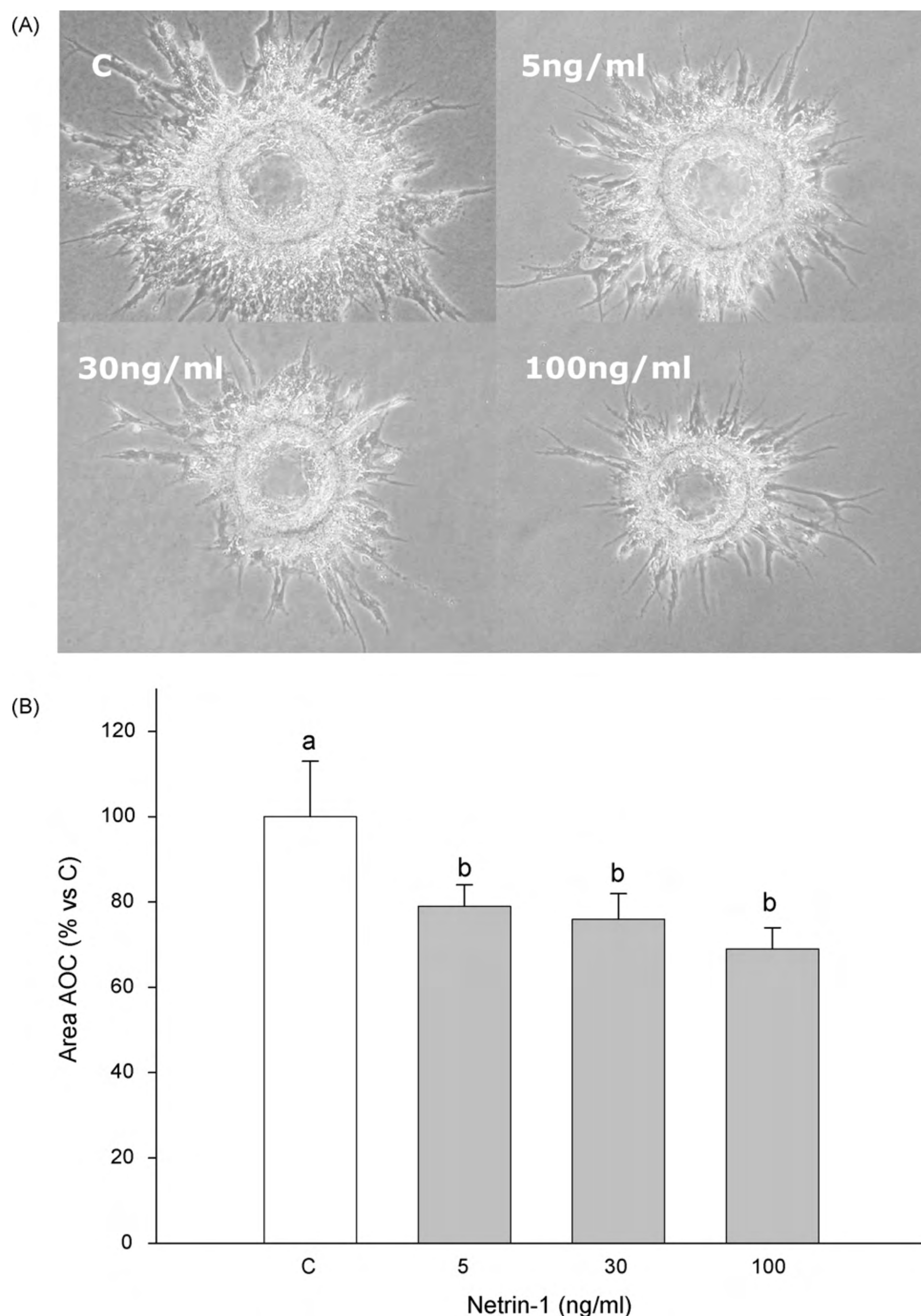


Fig. 8. Effect of the 96 h treatment with or without (C) netrin-1 at the concentrations of 5, 30 or 100 ng/ml on AOC growth in fibrin gel. Panel (A) phase contrast micrographs showing AOC growth at 96 h in fibrin gel matrix. Panel (B) area covered by AOC. Data, expressed as % vs respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.05$).

important aspect of growth and metastasis of solid tumors (Grimm et al., 2009). The similarities between nerve and blood vessel guidance have attracted considerable interest towards the study of a potential involvement of neurotropic factors in angiogenesis control (Strizzi et al., 2008).

Among these, netrin-1 deserves particular attention even if its exact role in angiogenesis is still doubtful since numerous studies report both pro- or anti-angiogenic activities (Bradford et al., 2009). The results obtained in the fibrin bioassay demonstrate an inhibition of angiogenesis by netrin-1. This is in agreement with Lu et al. (2004), but contrary to Park et al. (2004) and Wilson et al. (2006) who suggested that netrin-1 stimulates new vessel growth. Therefore, it appears that netrin-1 function, as already demonstrated in the nervous system (Masuda et al., 2009), could be dependent on receptor type as well as on the intracellular signal transduction pathways activated by each receptor. Further studies will be needed in order to investigate this fundamental topic in our experimental model. Our data also suggest, for the first time, that this effect most likely appears to be mediated by an inhibition of VEGF production by endothelial cells. Moreover, our data are in agreement with Nguyen and Cai (2006) demonstrate that netrin-1 appears to increase NO output by endothelial cells. It should be noted that the role of NO in angiogenesis regulation is far from being clarified. Several studies suggest a stimulatory effect (Kimura et al., 2000; Dulak et al., 2000; Sandau et al., 2001) while others point out inhibitory effects (Powell et al., 2000; Santini et al., 2009). The reasons for these discrepancies are still unknown, even if NO levels could play an important role (Kimura and Esumi, 2003).

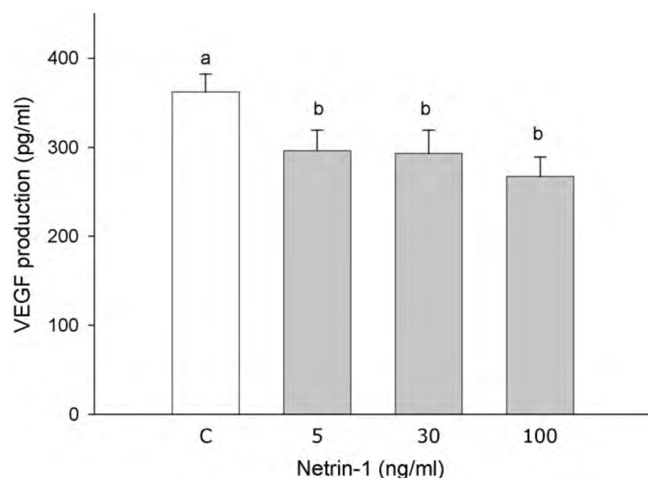


Fig. 9. Effect of the 48 h treatment with or without (C) netrin-1 at the concentrations of 5, 30 or 100 ng/ml on VEGF production, evaluated by ELISA, by AOC culture. Data, expressed as pg/ml, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.01$).

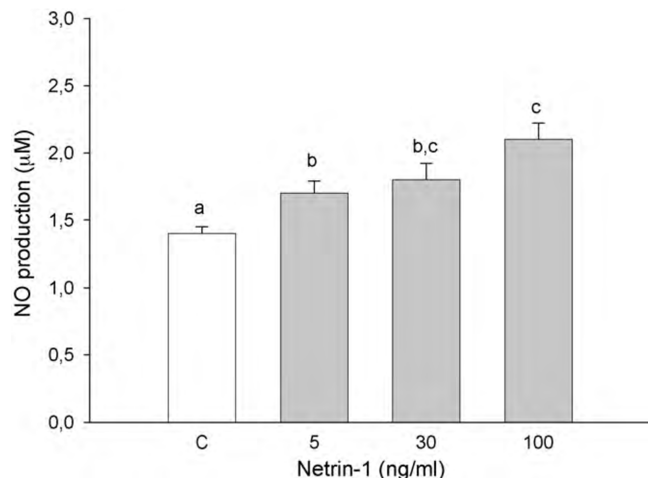


Fig. 10. Effect of the 48 h treatment with or without (C) netrin-1 at the concentrations of 5, 30 or 100 ng/ml on NO production, evaluated by Greiss method, by AOC culture. Data, expressed as μM, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. The absence of common letters indicates a significant difference ($p < 0.01$).

Taken together our data demonstrate that netrin-1 is physiologically present in swine ovary and it is likely involved in modulating follicular function. In particular, data obtained in granulosa cells let us to hypothesize that netrin-1 could be important for granulosa cell terminal differentiation; moreover, the observations in endothelial cells seem to suggest its involvement in the control of new vessel growth. Further studies are strongly advisable to address netrin-1 function since the molecular machinery responsible for angiogenesis inhibition is far to be clarified.

Acknowledgements

We would like to thank Professor Hinck, Department of Molecular, Cell and Developmental Biology, University of California, Santa Cruz, California and Professor Yelamos (Department of Biochemistry, Molecular Biology and Immunology, Facultad de Medicina, Universidad de Murcia, Spain) for supplying AOC. This research was supported by a FIL and MIUR PRIN grants.

References

Barboni, B., Turriani, M., Galeati, G., Spinaci, M., Bacci, M.L., Forni, M., Mattioli, M., 2000. Vascular endothelial growth factor production in growing pig antral follicles. Biol. Reprod. 63, 858–864.

Basini, G., Baratta, M., Bussolati, S., Tamanini, C., 1998. Interleukin-1 beta fragment (163–171) modulates bovine granulosa cell proliferation in vitro: dependence on size of follicle. J. Reprod. Immunol. 37, 139–153.

Basini, G., Bussolati, S., Santini, S.E., Bianchi, F., Careri, M., Mangia, A., Musci, M., Grasselli, F., 2008a. Hydroxyestrogens inhibit angiogenesis in swine ovarian follicles. J. Endocrinol. 199, 127–135.

Basini, G., Bussolati, S., Santini, S.E., Grasselli, F., 2008b. Reactive oxygen species and anti-oxidant defences in swine follicular fluids. Reprod. Fertil. Dev. 20, 269–274.

Basini, G., Baioni, L., Bussolati, S., Grolli, S., Kramer, L.H., Wagner, G.F., Grasselli, F., 2010. Expression and localization of stanniocalcin 1 in swine ovary. Gen. Comp. Endocrinol. 166, 404–408.

Bouvrée, K., Larrivée, B., Lv, X., Yuan, L., DeLafarge, B., Freitas, C., Mathivet, T., Bréant, C., Tessier-Lavigne, M., Bikfalvi, A., Eichmann, A., Pardanaud, L., 2008. Netrin-1 inhibits sprouting angiogenesis in developing avian embryos. Dev. Biol. 318, 172–183.

Bradford, D., Cole, S.J., Cooper, H.M., 2009. Netrin-1: diversity in development. Int. J. Biochem. Cell. Biol. 413, 487–493.

Carrello, S., Chamorro, M., Rodriguez-Gago, B., Alvarez, M.J., Molina, J.I., Rodriguez-Barbosa, A., Sanchez, P., Ramirez, A., Munoz, J., Dominguez, J., Parrilla, P., Yelamos, J., 2002. Isolation and characterization of immortalized porcine aortic endothelial cell lines. Vet. Immunol. Immunopathol. 89, 91–98.

Castets, M., Coissieux, M.M., Delloye-Bourgeois, C., Bernard, L., Delcros, J.G., Bernet, A., Laudet, V., Mehlen, P., 2009. Inhibition of endothelial cell apoptosis by netrin-1 during angiogenesis. Dev. Cell. 16, 614–620.

Cirulli, V., Yebra, M., 2007. Netrins: beyond the brain. Nat. Rev. Mol. Cell. Biol. 8, 296–306.

Dulak, J., Józkowicz, A., Dembinska-Kiec, A., Guevara, I., Zdzienicka, A., Zmudzinska-Grochot, D., Florek, I., Wójtowicz, A., Szuba, A., Cooke, J.P., 2000. Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 20, 659–666.

Grimm, D., Bauer, J., Schoenberger, J., 2009. Blockade of neoangiogenesis, a new and promising technique to control the growth of malignant tumors and their metastases. Curr. Vasc. Pharmacol. 7, 347–357.

Grasselli, F., Baratta, M., Tamanini, C., 1993. Effects of a GnRH analogue (buserelin) infused via osmotic minipumps on pituitary and ovarian activity of prepuberal heifers. Anim. Reprod. Sci. 32, 153–161.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E.D., Chan, S.S., Culotti, J.G., Tessier-Lavigne, M., 1996. Deleted in colorectal cancer (DCC) encodes a netrin receptor. Cell 87, 175–185.

Kimura, H., Esumi, H., 2003. Reciprocal regulation between nitric oxide and vascular endothelial growth factor in angiogenesis. Acta Biochim. Pol. 50, 49–59.

Kimura, H., Weisz, A., Kurashima, Y., Hashimoto, K., Ogura, T., D'Acquisto, F., Addeo, R., Makuuchi, M., Esumi, H., 2000. Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. Blood 95, 189–197.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Larrivée, B., Freitas, C., Trombe, M., Lv, X., Delafarge, B., Yuan, L., Bouvrée, K., Bréant, C., Del Toro, R., Bréchot, N., Germain, S., Bono, F., Dol, F., Claes, F., Fischer, C., Autiero, M., Thomas, J.L., Carmeliet, P., Tessier-Lavigne, M., Eichmann, A., 2007. Activation of the UNC5B receptor by Netrin-1 inhibits sprouting angiogenesis. Genes Dev. 21, 2433–2447.

Larrivée, B., Freitas, C., Suchting, S., Brunet, I., Eichmann, A., 2009. Guidance of vascular development: lessons from the nervous system. Circ. Res. 104, 428–441.

Lu, X., Le Noble, F., Yuan, L., Jiang, Q., De Lafarge, B., Sugiyama, D., Bréant, C., Claes, F., De Smet, F., Thomas, J.L., Autiero, M., Carmeliet, P., Tessier-Lavigne, M., Eichmann, A., 2004. The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. Nature 432, 179–186.

Maeda, A., Matsuda, F., Goto, Y., Cheng, Y., Gonda, H., Inoue, N., Nakagawa, S., Manabe, N., 2008. Molecular cloning of a porcine (Sus scrofa) apoptosis inhibitory ligand, netrin-1, and its receptor, p53RDL1. J. Reprod. Dev. 54, 275–280.

Masuda, T., Yaginuma, H., Sakuma, C., Ono, K., 2009. Netrin-1 signaling for sensory axons: Involvement in sensory axonal development and regeneration. Cell. Adhes. Migr. 3, 171–173.

Nguyen, A., Cai, H., 2006. Netrin-1 induces angiogenesis via a DCC-dependent ERK1/2-eNOS feed-forward mechanism. Proc. Natl. Acad. Sci. U.S.A. 103, 6530–6535.

Park, K.W., Crouse, D., Lee, M., Karnik, S.K., Sorensen, L.K., Murphy, K.J., Kuo, C.J., Li, D.Y., 2004. The axonal attractant Netrin-1 is an angiogenic factor. Proc. Natl. Acad. Sci. U.S.A. 101, 16210–16215.

Powell, J.A., Mohamed, S.N., Kerr, J.S., Mousa, S.A., 2000. Antiangiogenesis efficacy of nitric oxide donors. J. Cell. Biochem. 80, 104–114.

Sandau, K.B., Fandrey, J., Brüne, B., 2001. Accumulation of HIF-1alpha under the influence of nitric oxide. Blood 97, 1009–1015.

Santini, S.E., Basini, G., Bussolati, S., Grasselli, F., 2009. The phytoestrogen quercetin impairs steroidogenesis and angiogenesis in swine granulosa cells in vitro. J. Biomed. Biotechnol., 2009–419891.

Serafini, T., Kennedy, T.E., Galko, M.J., Mirzayan, C., Jessell, T.M., Tessier-Lavigne, M., 1994. The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. Cell 78, 409–424.

Please cite this article in press as: Basini, G., et al., The axonal guidance factor netrin-1 as a potential modulator of swine follicular function. Mol. Cell. Endocrinol. (2010), doi:10.1016/j.mce.2010.08.001

Strizzi, L., Mancino, M., Bianco, C., Raafat, A., Gonzales, M., Booth, B.W., Watanabe, K., Nagaoka, T., Mack, D.L., Howard, B., Callahan, R., Smith, G.H., Salomon, D.S., 2008. Netrin-1 can affect morphogenesis and differentiation of the mouse mammary gland. J. Cell. Physiol. 216, 824–834.
Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354.
Wilson, B.D., Ii, M., Park, K.W., Suli, A., Sorensen, L.K., Larrieu-Lahargue, F., Urness, L.D., Suh, W., Asai, J., Kock, G.A., Thorne, T., Silver, M., Thomas, K.R., Chien, C.B., Losordo, D.W., Li, D.Y., 2006. Netrins promote developmental and therapeutic angiogenesis. Science 313, 640–644.

ALLEGATO E

"Netrin-1: just an axon-guidance factor?”

Vet Res Commun. 2010. 34: S1-S4. doi: 10.1007/s112 59-010-9366-5.

Vet Res Commun
DOI 10.1007/s11259-010-9366-5

EXTENDED ABSTRACT

Netrin-1: Just an axon-guidance factor?

L. Baioni · G. Basini · S. Bussolati · C. Cortimiglia · F. Grasselli

© Springer Science+Business Media B.V. 2010

Abstract

Netrin-1 was first identified as a guidance factor in axon outgrowth during central nervous system development and was later shown to be involved in the morphogenesis of other organs. This study, thus, aimed to verify netrin-1 gene expression in swine antral follicles and to detect netrin-1 protein expression in follicular fluid. In addition, since netrin-1 is also a potential guidance factor for endothelial cells during angiogenesis, an essential event for follicular development, we attempted to verify its effects on swine aortic endothelial cells. Our results show that netrin-1 is present in follicular fluid and is physiologically expressed in both the thecal and granulosa layers from swine antral follicles. Furthermore, by means of an angiogenesis bioassay, we documented the inhibition of vascular neoformation by netrin-1. In conclusion, our results demonstrate that netrin-1 can be synthesized by swine follicular cells and secreted in the follicular fluid where it appears to exert regulatory effects on both follicular function and vascular development.

Keywords Angiogenesis · Netrin-1 · Ovary

Abbreviations

AOC	aortic endothelial cell
DAB	3,3′ diaminobenzidine
FCS	fetal calf serum
MC	microcarriers
PBS	phosphate buffered saline
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TTBS	tris-buffered saline plus tween
UNC5B	uncoordinated 5B
PCR	polymerase chain reaction

L. Baioni · G. Basini (✉) · S. Bussolati · C. Cortimiglia · F. Grasselli
Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti, Facoltà di Medicina Veterinaria, Università degli Studi di Parma, Via del Taglio 10, 43100 Parma, Italy
e-mail: basini@unipr.it

Introduction 47

In 1994, Kennedy and collaborators discovered netrin-1 (Kennedy et al. 1994), a factor that guides axon pathfinding during the development of the central nervous system. Netrin-1 involvement in the morphogenesis of other organs (mammary gland, pancreas, and lung) has been documented previously, and its expression has been demonstrated in the heart, liver, kidney, intestine, stomach, thymus, and testicles (Engelkamp 2002). At present, the only experimental evidence of the protein within the ovary is related to swine granulosa cells (Maeda et al. 2008). Therefore, the first aim of this study was to verify netrin-1 expression in swine ovarian follicles greater than 5 mm, in both the theca and granulosa layer. In addition, we tried to verify its presence in the follicular fluid by Western blot. Recent studies show that netrin-1 is able to drive pathfinding of not only neurons, but also of endothelial cells during blood vessel development (Wilson et al. 2006). In particular, Lu and co-workers (Lu et al. 2004) have shown that netrin-1 mediates repulsive guidance in the growth of the vascular network through its binding to the UNC5B receptor. In contrast, Park and colleagues (Park et al. 2004) report a promoter effect on both migration and proliferation. Therefore, using an angiogenesis bioassay (Basini et al. 2008), we examined the effects of netrin-1 on the development of new blood vessels. 48–63

Materials and methods 64

Swine ovaries were obtained from a slaughterhouse, and antral follicles greater than 5 mm were isolated. Granulosa cells were separated from the thecal membrane, and RNA was extracted and reverse-transcribed. The cDNA obtained was subjected to PCR using the primers pACTIN sense (5′-GAGACCTTCAACACGCCG-3′) and antisense (5′-GGAAGGTGGA CAGCGAGG-3′), with an expected product size of 685 bp; and netrin-1 forward (5′-GACG ACGACGAGGAGAACTC-3′) and reverse (5′-GGGTGAGCTCAATCACAGTT-3′), which amplify a region of 449 bp. The amplified products obtained were subjected to electrophoresis in a 1.5% agarose gel stained with Gel Red dye (Biotium, Hayward, CA). Digital images were captured by a Power Shot A610 (Canon Italy SpA, Milan). Follicular fluid, collected by aspiration, was subjected to SDS-PAGE and subsequently blotted onto a ProBlott membrane. After transfer, the membrane was incubated in a solution of 5% skim milk and TBS for 1 hour. After three washes in TTBS, the membrane was incubated with an anti-netrin-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For immune complex detection, we used a universal biotinylated secondary antibody (LSAB ® + System-HRP, Dako Cytomation, Carpinteria, CA) in combination with streptavidin-peroxidase and DAB as chromogen substrate. 65–80

The in vitro angiogenesis bioassay was performed by incubating 12.5 mg MC with 1.5 mL sterile PBS for 3 hours at 37°C. After two washes, the MC were put in a flask containing AOC (5×10^5) at the 13th passage, in 5 mL M199 containing 2% FCS. The flask was incubated overnight at 37°C to let the AOC coat the MC. For fibrin gel preparation, the AOC-coated MC (20 μL) were placed in 12-well plates containing a solution of fibrinogen 81–85

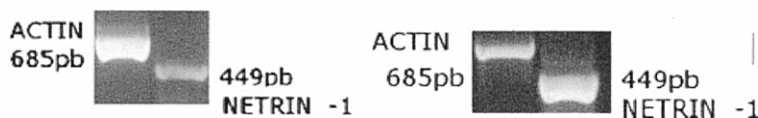


Fig. 1 Netrin-1 expression (and control actin expression) in granulosa cells (left) and theca cells (right) from large follicles

Fig. 2 The ProBlott membrane shows a band of approximately 70 kDa, identifiable as netrin-1

(1 mg/mL, pH 7.6) and thrombin (5 U/mL). The plates were incubated for 30 min at 37°C to promote fibrin polymerization. Subsequently, 1 mL M199 was added to each well. After a change of medium, fibrin gels were treated with 100 ng/mL netrin-1 (kindly provided by Prof. Linsday Hink, University of California, Santa Cruz, CA) and incubated for 48 hours at 37°C in 5% CO_2. Photographic images were acquired after incubation to determine the area of vascular development. AOC proliferation in the fibrin gel was quantified using Scion Image 4.02 (Scion Corporation, Frederick, MA, http://rsb.info.nih.gov/nih-image/), which calculates the area occupied by endothelial cells expressed as the number of pixels. Statistical differences were calculated by ANOVA using Statgraphics software (STC Inc., Rockville, MD). When statistically significant differences were found ($p<0.05$), means were submitted to the Scheffè's F test for multiple comparisons. 86 87 88 89 90 91 92 93 94 95 96

Results 97

Netrin-1 gene expression was detected in both theca and granulosa cells isolated from large antral follicles (Fig. 1). Immunoblotting revealed the presence of netrin-1 in follicular fluid (Fig. 2). 98 99 100

Netrin-1 treatment produced a significant inhibition ($p<0.05$) of angiogenesis in the area covered by endothelial cells on the fibrin gel (Fig. 3). 101 102

Discussion 103

Our results show that netrin-1 is present in follicular fluid and is physiologically expressed in both theca and granulosa cells of swine antral follicles larger than 5 mm, suggesting its involvement in regulating follicular function. Netrin-1 appears to be primarily expressed in regions of tissue remodelling rather than in quiescent tissue (Strizzi et al. 2008). Interestingly, the ovarian follicle is a typical site of differentiation and reorganization 104 105 106 107 108

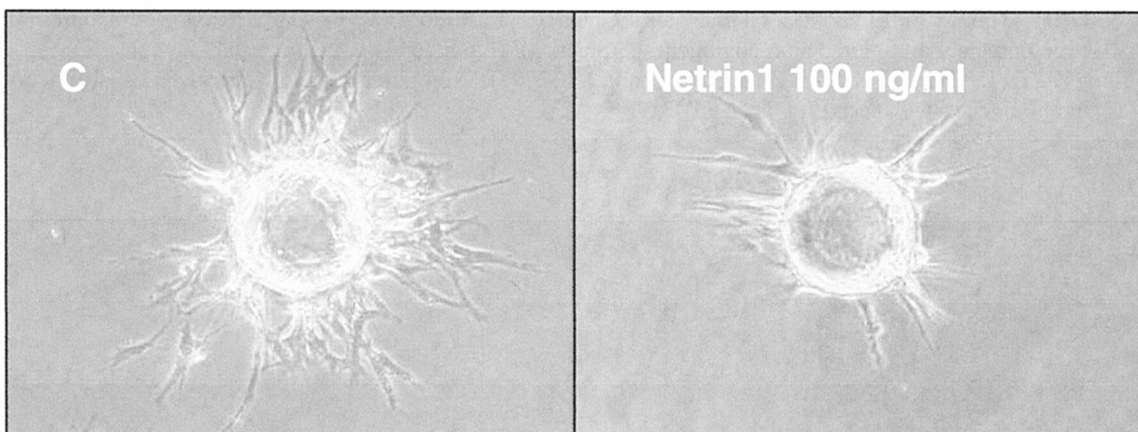


Fig. 3 Phase-contrast micrographs showing the effects of 100 ng/mL netrin-1 on AOC growth after 48 hours; C: control (no netrin-1)

events, leading to the evolution of both its structure and the thecal vascular system. Therefore, netrin-1 could be actively involved in driving such dynamic events. Based on our data, we hypothesize that netrin-1 can be synthesized locally and could contribute both to the organization of follicle cells and to the regulation of blood network development, an essential event during follicular maturation. Specifically, using an angiogenesis bioassay, we documented the inhibition of vascular formation by netrin-1. This finding indicates that netrin-1 is an anti-angiogenic factor. Consistent with the data obtained in the nervous system where netrin-1 exerts opposing effects that depend on the type of receptor involved, previous studies documented that this protein can either inhibit or promote angiogenesis. In particular, Yang and colleagues (2007) showed that netrin-1 might act as a pro- or anti-angiogenic factor, depending on its concentration and the type of receptor expressed by endothelial cells. In conclusion, this protein, which is known as an axon-guidance factor, can be synthesized by swine follicular cells and secreted into the follicular fluid where it can exert regulatory effects on neovascularization.

Acknowledgements This work was supported by MIUR-PRIN and FIL grants.

References

Basini G, Bussolati S, Santini SE, Bianchi F, Careri M, Mangia A, Musci M, Grasselli F (2008) Hydroxyestrogens inhibit angiogenesis in swine ovarian follicles. Endocrinology 199:127–135
Engelkamp D (2002) Cloning of three mouse Unc5 genes and their expression patterns at mid-gestation. Mech Dev 118:191–197
Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M (1994) Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell 78:425–435
Lu X, Le Noble F, Yuan L, Jiang Q, De Lafarge B, Sugiyama D, Bréant C, Claes F, De Smet F, Thomas JL, Autiero M, Carmeliet P, Tessier-Lavigne M, Eichmann A (2004) The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. Nature 432:179–186
Maeda A, Matsuda F, Goto Y, Cheng Y, Gonda H, Inoue N, Nakagawa S, Manabe N (2008) Molecular cloning of a porcine (*Sus scrofa*) apoptosis inhibitory ligand, netrin-1, and its receptor, p53RDL1. J Reprod Dev 54:275–280
Park KW, Crouse D, Lee M, Karnik SK, Sorensen LK, Murphy KJ, Kuo CJ, Li DY (2004) The axonal attractant netrin-1 is an angiogenic factor. Proc Natl Acad Sci USA 101:16210–16215
Strizzi L, Postovit LM, Margaryan NV, Seftor EA, Abbott DE, Seftor RE, Salomon DS, Hendrix MJ (2008) Emerging roles of nodal and Cripto-1: From embryogenesis to breast cancer progression. Breast Dis 29:91–103
Wilson BD, Ii M, Won Park K, Suli A, Sorensen LK, Larrieu-Lahargue F, Urness LD, Suh W, Asai J, Kock GAH, Thorne T, Silver M, Thomas KR, Chien C, Losordo DW, Li DY (2006) Netrins promote developmental and therapeutic angiogenesis. Science 313:640–644
Yang Y, Zou L, Wang Y, Xu KS, Zhang JX, Zhang JH (2007) Axon guidance cue Netrin-1 has dual function in angiogenesis. Cancer Biol Ther 6:743–748

ALLEGATO F

"Gossypol, a polyphenolic aldeide from cotton plant, interferes with swine granulosa cell function"

Domestic Animal Endocrinology. 2009. 37:30-36.
doi:10.1016/j.domaniend.2009.01.005.

Available online at www.sciencedirect.com
ScienceDirect
Domestic Animal Endocrinology xxx (2009) xxx–xxx
DOMESTIC ANIMAL ENDOCRINOLOGY
www.domesticanimalendo.com

Gossypol, a polyphenolic aldehyde from cotton plant, interferes with swine granulosa cell function

G. Basini *, S. Bussolati, L. Baioni, F. Grasselli

Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti - Sezione di Fisiologia Veterinaria, Via del Taglio 10, Università degli Studi di Parma, 43100 Parma, Italy

Received 19 September 2008; received in revised form 23 December 2008; accepted 15 January 2009

Abstract

Gossypol is a polyphenol isolated from the seed, roots and stem of cotton plant (*Gossypium sp.*) It has been associated with adverse effects on female reproduction, but recently also shown having promising effects against several malignancies. Its mechanisms of action are however still not fully understood. This study was therefore conducted to investigate the effect of 5 or 25 μg/ml gossypol on swine granulosa cell steroidogenic activity, redox status and Vascular Endothelial Growth Factor (VEGF) production. Study demonstrated that gossypol significantly ($p < 0.001$) inhibited granulosa cell estradiol 17β and progesterone production, an effect that could be at least partially mediated by an increase ($p < 0.05$) of nitric oxide and superoxide anion production as a consequence of superoxide dismutase inhibition. Moreover, gossypol stimulates ($p < 0.001$) VEGF production. In conclusion, study has demonstrated effecs of gossypol on swine granulosa cell function in vitro. Effects on female swine fertility can not be excluded.
© 2009 Published by Elsevier Inc.

Keywords: Angiogenesis; Ovary; Free radicals; Steroids; Reproduction

1. Introduction

Gossypol ($C_{30}H_{30}O_8$) is an aldehyde that is produced in the pigment glands of the roots, leaves, stems and seeds of the cotton plant genus *Gossypium*. Gossypol and two additional tautomeric forms, the hemiacetal and phenolic ketonoid, have been demonstrated to account for numerous chemical and biological reactions associated with this compound [1]. During the early 1900s, toxicosis caused by excessive consumption of cotton products by non-ruminant animals was related to gossypol poisoning. In the 1960s, villagers of many Chinese rural areas had switched from cooking with soybean oil to crude cottonseed oil [2]. Several years later many couples experienced fertility problems [3]. A similar impairment of reproductive performances has been documented in dairy cows fed with cottonseed and its by-products. In particular, reduced rates of pregnancy and increased pregnancy loss have been related to high free gossypol diets [4]. A negative effect of gossypol on fertility has also been shown in non ruminant females such as rats and hamsters, possibly by a disruption of steroid hormone metabolism [5] as well as cytotoxicity in the embryo [6,7]. In the pig, symptoms of chronic ingestion of high levels of free gossypol in cottonseed meal include labored breathing, dyspnea, decreased growth rate and anorexia [8]. However, the effects of gossypol on swine reproduction have not been studied in detail. However, a few published data indicate that gossypol interferes with the expression of normal estrous cycle probably due to mechanisms that affect ovarian func-

* Corresponding author. Tel.: +39 0521 032775; fax: +39 0521 032770.
E-mail address: basini@unipr.it (G. Basini).

0739-7240/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.domaniend.2009.01.005

UNCORRECTED PROOF

tion. Moreover, administration of this substance before implantation would inhibit pregnancy [9]. In order to gain more detailed knowledge on the effect of gossypol on female reproduction and to unravel its mechanisms of action, we examined its effects on swine granulosa cell proliferation and steroidogenesis, the main parameters of ovarian cell function. In addition, being aware of the fundamental role of reactive oxygen species in mediating follicular physiology, the effects of gossypol on granulosa cell redox status were examined. Finally, since we have previously demonstrated [10] that angiogenesis is essential for ovarian follicle development, we also tested a possible modulatory action on the production of the main proangiogenic factor, VEGF, by granulosa cells.

2. Materials and methods

All reagents used in this study were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

2.1. Granulosa cell collection

Swine ovaries were collected at a local slaughterhouse from Large White cross-bred gilts, parity = 0. The stage of the cycle were unknown. Follicles were classified on a dimension-based fashion [11]. The ovaries were placed into cold PBS (4 °C) supplemented with penicillin (500 IU/ml), streptomycin (500 μg/ml) and amphotericin B (3.75 μg/ml), maintained in a freezer bag and transported to the laboratory within 1 h. After a series of washings with PBS and ethanol (70%) granulosa cells were aseptically harvested by aspiration of large follicles (> 5 mm) with a 26-gauge needle and released in medium containing heparin (50 IU/ml), centrifuged for pelleting and then treated with 0.9% prewarmed ammonium chloride at 37 °C for 1 min to remove red blood cells. Cell number and viability were estimated using a haemocytometer under a phase contrast microscope after vital staining with trypan blue (0.4%) of an aliquot of the cell suspension. Cells were seeded in culture medium (CM) M199 supplemented with sodium bicarbonate (2.2 mg/ml), bovine serum albumin (BSA 0.1%), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), selenium (5 ng/ml) and transferrin (5 μg/ml). Once seeded, cells were incubated in the presence or absence of gossypol (5 or 25 μg/ml) and maintained for 48 h at 37 °C under humidified atmosphere (5% CO_2). This procedure was identical for all experiments performed in this study.

2.2. Granulosa cell proliferation and steroid production

2.2.1. Cell proliferation

10^4 cell/well were seeded in 96-well plates in 200 μl CM. Cell proliferation was evaluated by 5-bromo-2′-deoxyuridine (BrdU) incorporation assay test (Roche, Mannheim, Germany). Briefly, after addition of 20 μl BrdU to each well after 44 h of incubation in the presence or absence of gossypol treatments, culture media were removed and a DNA denaturating solution was added in order to improve the accessibility of the incorporated BrdU for antibody detection. Thereafter, 100 μl anti-BrdU antibody were added to each well. After a 1.5 h incubation at room temperature, the immune complexes were detected by the subsequent substrate reaction. The reaction product was quantified by measuring the absorbance at a wavelength of 450 nm against 690 nm using a Spectra Shell Microplate reader (SLT Spectra, Milan, Italy). To test the viable cell number, absorbance was related to a standard curve prepared by culturing in quintuplicate granulosa cells at different plating densities (from 10^3 to 10^5/200 μl) for 48 h. The curve was repeated in four different experiments. The relationship between cell number and absorbance was linear (r = 0.92). Cell number/well was estimated from the resulting linear regression equation. The assay detection limit was 10^3 cell/well and the variation coefficient was less than 5%. The number of cells obtained was used for correcting hormones, VEGF production and redox status data.

2.2.2. Steroid production

10^4 cells/well were seeded in 96-well plates in 200 μl CM supplemented with androstenedione (28 ng/ml). Culture media were then collected, frozen and stored at –20 °C until progesterone (P4) and 17β estradiol (E2) determination by validated RIAs [12].

P4 assay sensitivity and ED50 were 0.24 and 1 nmol/l, respectively; E2 assay sensitivity and ED50 were 0.05 and 0.2 nmol/l. The intra- and inter-assay coefficients of variation were less than 12% for both assays.

2.3. Granulosa cell redox status

2.3.1. NO production

10^5 cells/200 μl CM were seeded in 96-well plates. NO was assessed by measuring nitrite levels in culture media by the microplate method based on the formation of chromophore after reaction with Griess reagent, which was prepared fresh daily by mixing equal volumes of stock A (1% sulfanilamide, 5% phosphoric acid)

UNCORRECTED PROOF

Please cite this article in press as: Basini G, et al. Gossypol, a polyphenolic aldehyde from cotton plant, interferes with swine granulosa cell function. Domest Anim Endocrinol (2009), doi:10.1016/j.domaniend.2009.01.005

UNCORRECTED PROOF

and stock B (0,1% N-[naphthyl] ethylenediamine dihydrochloride) [13].

2.3.2. Superoxide dismutase activity

2×10^5 cells/200 µl CM were seeded in 96-well plates. SOD activity was determined by a SOD Assay Kit (Dojindo Molecular Technologies, Japan). After centrifugation for 10 min at 400 × g, the supernatants were discarded and cells were lysed adding cold Triton 1% in TRIS HCl (100 µl/10^5 cells) and incubating on ice for 30 min. Cell lysates were tested without dilution. A standard curve of SOD ranging from 0.156 to 20 U/ml was prepared. The assay measures formazan produced by the reaction between tetrazolium salt (WST-1) and superoxide anion (O_2^-), generated adding xantine oxidase. Endogenous SOD activity is related to the remaining O_2^-. The absorbance was determined with a Spectra Shell Microplate Reader reading at a wavelength of 450 nm against 620 nm.

2.3.3. WST-1 assay for superoxide (O_2^-) production

O_2^- production was evaluated by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) test (Roche, Mannheim, Germany). The assay is based on the cleavage of the water-soluble tetrazolium salt, WST-1 to a yellow-orange, watersoluble formazan. Evidence exists that tetrazolium salts can be used as a reliable measure of intracellular O_2^- production [14,15]. 10^4 cells/200 µl CM were seeded in 96-well plates. 20 µl WST-1 were added to cells during the last 4 h of incubation and absorbance was then determined using a Spectra Shell Microplate reader at a wavelength of 450 nm against 620 nm.

2.3.4. Scavenging non-enzymatic activity: ferric reducing antioxidant power (FRAP) assay

FRAP assay is a colorimetric method based on the ability of the antioxidant molecules to reduce ferric-tripiridyltriazine (Fe^{3+}TPTZ) to a ferrous form (Fe^{2+}TPTZ). Fe^{2+} is measured spectrophotometrically via determination of its coloured complex with 2,4,6-Tris(2- pyridyl)-s-triazine (Fe^{2+} TPTZ). TPTZ reagent was prepared before use, mixing 25 ml of acetate buffer, 2.5 ml of 2,4,6-Tris(2- pyridyl)-s-triazine (TPTZ) 10 mmol/l in HCl 40 mmol/l and $FeCl^{3-}$ 6 H_2O solution.

Briefly, 2×10^5 cells/200 µl CM were seeded in 96-well plates. At the end, plates were centrifuged for 10 min at 400 × g, supernatants were discarded and cells were lysed adding cold Triton 0.5% + phenylmethylsulphonyl fluoride (PMSF) in PBS (200 µl per well), incubating on ice for 30 min. The test was performed on 40 µl of cell lysates added to Fe^{3+}TPTZ reagent and then incubated at 37 °C for 30 min. The absorbance of Fe^{2+} TPTZ was determined by Spectra Shell Microplate Reader at a wavelength of 595 nm. The ferric reducing ability of cell lysates was calculated by plotting a standard curve of absorbance against $FeSO^{4-}7H_2O$ standard solution.

2.4. Granulosa cell angiogenic activity

2.4.1. VEGF production

10^6 granulosa cells in 1 ml CM + 1% FCS were seeded in 24-well plates. VEGF in culture media was quantified by an ELISA (Quantikine, R&D System, Minneapolis, MI, USA). This assay, developed for human VEGF detection, has been validated for pig VEGF [16]. The assay sensitivity was 8.74 pg/ml, the inter- and intra-assay CVs were always less than 7%. A Spectra Shell microplate reader set to read at a wavelength of 450 nm emission was used to quantify the reaction product.

2.5. Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed by means of ANOVA using Statgraphics package (STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by Scheffè's F test. P values < 0.05 were considered to be statistically significant.

3. Results

3.1. Granulosa cell proliferation and steroid production

3.1.1. Cell proliferation

Gossypol significantly increased ($p < 0.01$) granulosa cell growth. The concentrations used exerted the same effect ($p < 0.01$; Fig. 1).

3.1.2. Steroid production

E2 and P4 basal production by granulosa cells were 3.7 ± 0.6 and 71.8 ± 10.0 ng/ml, respectively. Gossypol markedly inhibited ($p < 0.001$) steroidogenesis in granulosa cells. The effect was dose-dependent for both steroids (Fig. 2 A and B).

3.2. Granulosa cell redox status

3.2.1. NO production

In the control group, NO levels were 3.5 ± 0.3 µM. A significant increase was induced by 25 µg/ml gossy-

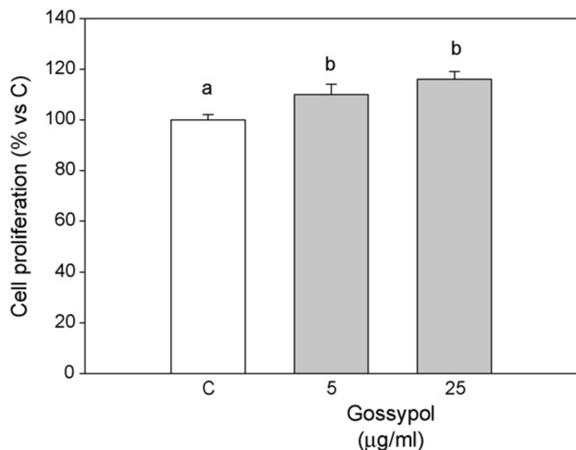


Fig. 1. Effect of the 48 h treatment with or without (C) gossypol (5 or 25 µg/ml) on granulosa cell proliferation using 5-bromo-2′-deoxyuridine (BrdU) incorporation assay test. Data, expressed as % vs respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.01$).

pol ($p < 0.05$), but not with the lowest concentration of 5 µg/ml gossypol (Fig. 3).

3.2.2. Superoxide dismutase activity

Basal superoxide dismutase activity (320 ± 10 mU/ml) was significantly ($p < 0.001$) inhibited by the treatment with 25 µg/ml of gossypol; on the contrary, the lowest concentration was ineffective as shown in Fig. 4.

3.2.3. WST-1 assay for superoxide (O_2^-) production

The highest gossypol concentration significantly stimulated ($p < 0.05$) O_2^- generation while the lowest concentration was ineffective (Fig. 5).

3.2.4. Scavenging non-enzymatic activity: FRAP assay

The antioxidant power was significantly ($p < 0.001$) increased by both concentrations of gossypol; 25 µg/ml resulted in the highest increase ($p < 0.001$; Fig. 6).

Fig. 7.

3.3. Granulosa cell angiogenic activity

3.3.1. VEGF production

960 ± 42 pg/ml VEGF were produced by granulosa cell in basal conditions. The lowest gossypol concentration significantly ($p < 0.001$) stimulated VEGF output, while the highest one was ineffective (Fig. 5).

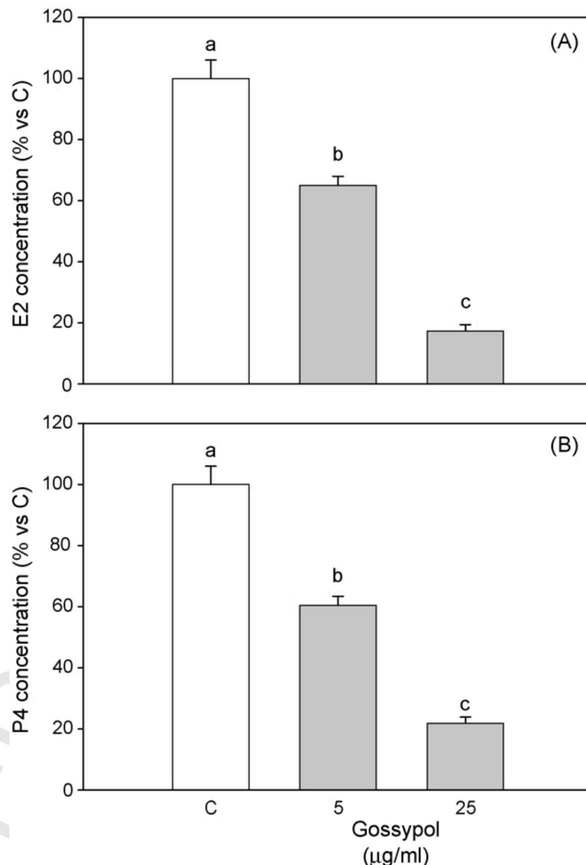


Fig. 2. Effect of the 48 h treatment with or without (C) gossypol (5 or 25 µg/ml) on E2 (A) and P4 (B) concentration in swine granulosa cell culture using RIA. Data, expressed as % vs respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.001$).

4. Discussion

Gossypol is a polyphenolic compound aldehyde derived from the cotton plant belonging to the family Malvaceae. Though it has been shown to negatively affect reproductive function in females of different mammalian species [7] research done so far is patchy and not always conclusive. Since there is recent evidence that gossypol may be a promising agent in the treatment of leukaemia [17], lymphoma [18] and of other malignancies [19–21], a deeper understanding of its molecular mechanisms of action is worthwhile. The results of this study show that gossypol stimulates swine granulosa cell proliferation. This is in contrast with data demonstrating gossypol antiproliferative effects on a variety of cancer cell lines [22]. The reason for this discrepancy may be that in this study gossypol was tested using normal primary cells, as done in the investigation of Akira et al [23]

UNCORRECTED

Please cite this article in press as: Basini G, et al. Gossypol, a polyphenolic aldehyde from cotton plant, interferes with swine granulosa cell function. Domest Anim Endocrinol (2009), doi:10.1016/j.domaniend.2009.01.005

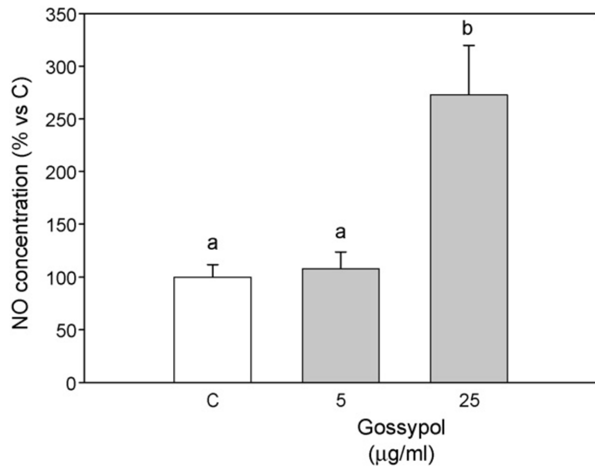


Fig. 3. Effect of the 48 h treatment with or without (C) gossypol (5 or 25 μg/ml) on NO concentration in swine granulosa cell culture using Griess reagent. Data, expressed as % vs respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.05$).

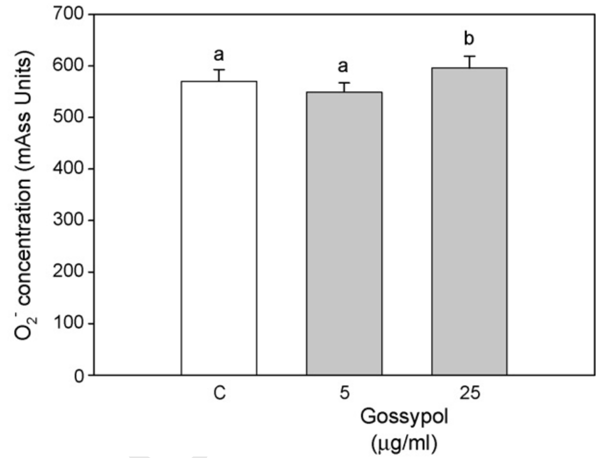


Fig. 5. Effect of the 48 h treatment with or without (C) gossypol (5 or 25 μg/ml) on O_2^- concentration in swine granulosa cell culture using WST-1 test. Data, expressed as milliAss Units, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.05$).

who did also not observe an inhibiting effect of gossypol on granulosa cell. It should be noted that gossypol kills cells specifically which is based on a genetically determined pathway and can vary between different cell types [24].

Several investigators have reported that gossypol treatment disrupts the normal pattern of estrous cycle in cows [25] and rats [26]; this effect might be the result of an impairement of steroidogenic pathway as evidenced in our present study. The molecular mechanism underlying this action is not clear. Gu et al [27] reported that inhibition of bovine luteal cell steroidogenesis by gossypol may be due to its effect on adenylate cyclase and 3β-hydroxysteroid dehydrogenase. On the other hand, gossypol was shown to interfere with key steroidogenic enzymes such as 5α-reductase and 3α-hydroxysteroid dehydrogenase in the rat testes [28] and to inhibit 5α-reductase in the canine prostate gland [29]. The present

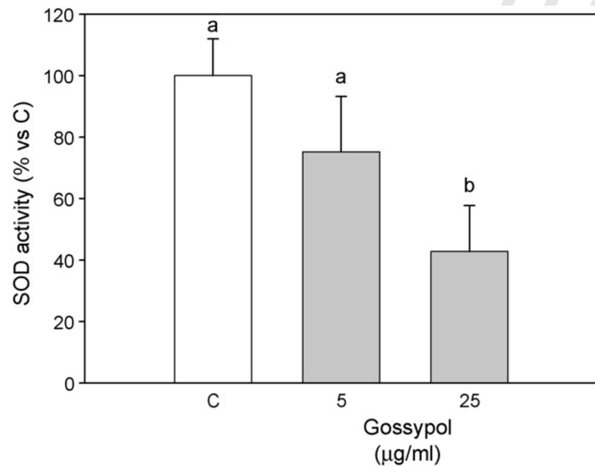


Fig. 4. Effect of the 48 h treatment with or without (C) gossypol (5 or 25 μg/ml) on swine granulosa cell SOD activity using colorimetric method. Data, expressed as % vs respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.001$).

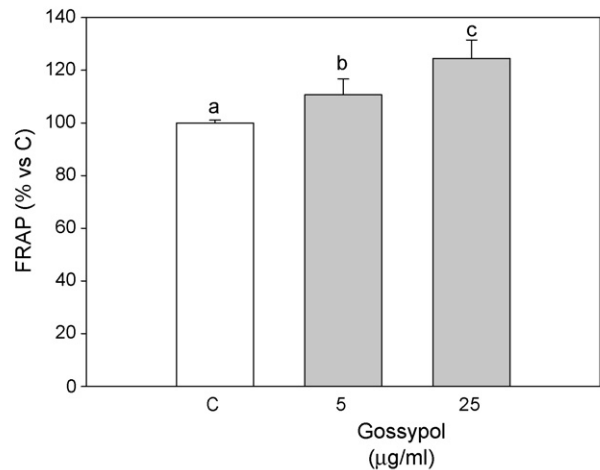


Fig. 6. Effect of the 48 h treatment with or without (C) gossypol (5 or 25 μg/ml) on FRAP levels in swine granulosa cell culture using colorimetric method. Data, expressed as % vs respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.001$).

Please cite this article in press as: Basini G, et al. Gossypol, a polyphenolic aldehyde from cotton plant, interferes with swine granulosa cell function. Domest Anim Endocrinol (2009), doi:10.1016/j.domaniend.2009.01.005

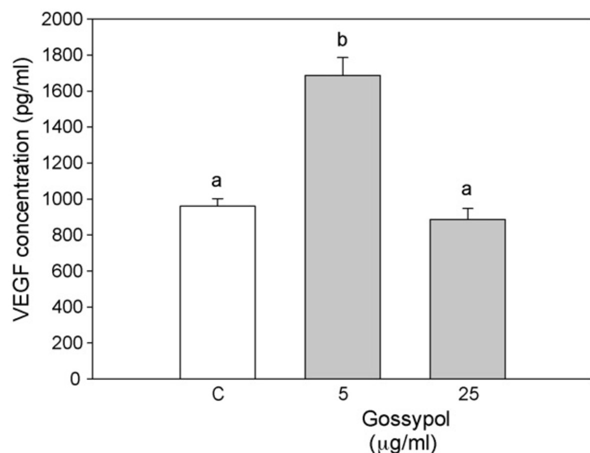


Fig. 7. Effect of the 48 h treatment with or without (C) gossypol (5 or 25 μg/ml) on VEGF concentration in swine granulosa cell culture using ELISA. Data, expressed as pg/ml, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p < 0.001$).

results demonstrate that the higher gossypol concentration stimulates nitric oxide (NO) production. Since this free radical has been shown to modulate ovarian steroidogenesis [30,31], due, at least in part to inhibition of steroid production [32] involving the second messenger cGMP [33–35], an involvement of NO in the gossypol-induced effects is assumed.

ROS have been indicated as participants in a variety of intracellular signalling sequences [36,37]. Gossypol is particularly interesting since it exhibits both pro- and anti-oxidant properties. This peculiar behaviour pertains to different substances that are commonly categorized as antioxidants, namely thiols, vitamin C and vitamin E [38]. Our results show that the higher gossypol concentration stimulates O_2^- generation by inhibiting SOD activity. Our results are consistent with the hypothesis that the increased O_2^- levels could play some role in the reduction of steroid production because they have been demonstrated to exert antisteroidogenic effects in granulosa cells [9]. On the contrary, both gossypol concentrations appear to stimulate FRAP levels which are indicators of the non-enzymatic radical scavengers, such as those represented by vitamins C, E and uric acid [39].

Gossypol is now considered a promising substance to contrast neoplasia development [40]. To the best of our knowledge, the effect of gossypol alone on tumour angiogenesis has never been examinated. Xu et al. [41] demonstrated that gossypol improves the outcome of cancer radiotherapy by increasing apoptosis mechanisms. The effect of gossypol on VEGF production has been investigated for the first time in present research. We demonstrate that this substance can stimulate the production of the proangiogenic peptide by granulosa cells. At the moment, no data have been published about the effect of gossypol on endothelial cell VEGF production. Recently, several papers [42–45] demonstrate that gossypol stimulates apoptotic events in cancer cells mainly by a direct inhibition of antiapoptotic Bcl-2 family proteins, Bcl-2 and Bcl-xL. Our present results suggest that the inhibitory effect of gossypol on E2 synthesis might result in negative effect of gossypol on E2 sinthesis might result in negative effects on E2-dependent tumours. Further studies will be required to better investigate this important topic.

Taken together, present research evidence that gossypol affect swine granulosa cell activity in vitro thus suggesting that this substance could interfere on female swine fertility.

Acknowledgements

This research was supported by a FIL grant.

References

[1] Bertrand JA, Sudduth TQ, Condon A, Jenkins TC, Calhoun MC. Nutrient content of whole cottonseed. J Dairy Sci 2005;88:1470–7.
[2] Liu BS. A tentative idea of the use of cooking cottonseed oil for fertility control. Shangai J Chin Med 1957;6:43–7.
[3] Liu GZ, Segal S.J. Introduction and history of gossypol. In: S.J. Segal (Ed.), Gossypol. A potential contraceptive for men, Plenum Press, New York 1-7.
[4] Santos JE, Villasenor M, Robinson PH, DePeters EJ, Holmberg CA. Type of cottonseed and level of gossypol in diets of lactating dairy cows: plasma gossypol, health, and reproductive performance. J Dairy Sci 2003;86:892–905.
[5] Wang NG, Zhou LF, Guan MH, Lei HP. Effect of (−)- and (+)-gossypol on fertility in male rats. J Ethnopharmacol 1987;20:21–4.
[6] Li YF, Booth GM, Seegmiller RE. Evidence for embryotoxicity of gossypol in mice and chicks with no evidence of mutagenic activity in the Ames test. Reprod Toxicol 1989;3(1):59–62.
[7] Randel RD, Chase Jr CC, Wyse SJ. Effects of gossypol and cottonseed products on reproduction of mammals. J Anim Sci 1992;70:1628–38.
[8] Fomband RB, Bryant MJ. An evaluation of the use of cottonseed caje in the diet of growing pigs. Trop Anim Health Prod 2004;36:295–305.
[9] Basini G, Bianco F, Grasselli F, Tirelli M, Bussolati S, Tamanini C. The effects of reduced oxygen tension on swine granulosa cell. Regul Pept 2004;120:69–75.
[10] Bianco F, Basini G, Grasselli F. Angiogenic activity of swine granulosa cells: effects of hypoxia and vascular endothelial growth factor Trap R1R2, a VEGF blocker. Domest Anim Endocrinol 2005;28:308–19.
[11] Basini G, Bussolati S, Santini S, Bianchi F, Careri M, Mangia A, et al. Hydroxyestrogens inhibit angiogenesis in swine ovarian follicles. J Endocrinol 2008;199:127–35.

Please cite this article in press as: Basini G, et al. Gossypol, a polyphenolic aldehyde from cotton plant, interferes with swine granulosa cell function. Domest Anim Endocrinol (2009), doi:10.1016/j.domaniend.2009.01.005

UNCORRECTED PROOF

[12] Grasselli F, Baratta M, Tamanini C. Effects of a GnRH analogue (buserelin) infused via osmotic minipumps on pituitary and ovarian activity of prepuberal heifers. Anim Reprod Sci 1993;32:153–61.
[13] Dong YL, Yallampalli C. Interaction between nitric oxide and prostaglandin E2 pathways in pregnant rat uteri. Am J Physiol 1996;270:471–6.
[14] Benov L, Fridovich I. Is reduction of the sulfonated tetrazolium 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2-tetrazolium 5-carboxanilide a reliable measure of intracellular superoxide production? Anal Biochem 2002;310:186–90.
[15] Ukeda H, Shimamura T, Tsubouchi M, Harada Y, Nakai Y, Sawamura M. Spectrophotometric assay of superoxide anion formed in Maillard reaction based on highly water-soluble tetrazolium salt. Anal Sci 2002;18:1151–4.
[16] Barboni B, Turriani M, Galeati G, Spinaci M, Bacci ML, Forni M, et al. Vascular endothelial growth factor production in growing pig antral follicles. Biol Reprod 2000;63:858–64.
[17] Balakrishnan K, Wierda WG, Keating MJ, Gandhi V. Gossypol, a BH3 mimetic, induces apoptosis in chronic lymphocytic leukemia cells. Blood 2008. Jun 19. [Epub ahead of print].
[18] Johnson PW. New targets for lymphoma treatment. Ann Oncol 2008;19:56–9.
[19] Badawy SZ, Souid AK, Cuenca V, Montalto N, Shue F. Gossypol inhibits proliferation of endometrioma cells in culture. Asian J Androl 2007;9:388–93.
[20] Ye W, Chang HL, Wang LS, Huang YW, Shu S, Dowd MK, et al. Modulation of multidrug resistance gene expression in human breast cancer cells by (−)-gossypol-enriched cottonseed oil. Anticancer Res 2007;27:107–16.
[21] Ko CH, Shen SC, Yang LY, Lin CW, Chen YC. Gossypol reduction of tumor growth through ROS-dependent mitochondria pathway in human colorectal carcinoma cells. Int J Cancer 2007;121:1670–9.
[22] Leblanc ML, Russo J, Kudelka AP, Smith JA. An in vitro study of inhibitory activity of gossypol, a cottonseed extract, in human carcinoma cell lines. Pharmacol Res 2002;46:551–5.
[23] Akira A, Ohmura H, Uzumcu M, Araki T, Lin YC. Gossypol inhibits aromatase activity in cultured porcine granulosa cells. Theriogenology 1994;41:1489–97.
[24] Qiu J, Levin LR, Buck J, Reidenberg MM. Different pathways of cell killing by gossypol enantiomers. Exp Biol Med 2002;227:398–401.
[25] Gray ML, Greene LW, Williams GL. Effects of dietary gossypol consumption on metabolic homeostasis and reproductive endocrine function in beef heifers and cows. J Anim Sci 1993;71:3052–9.
[26] Akinola OB, Oderinde OO, Adejumo AT, Bayode ED. Effect of cottonseed oil on oestrous cycle and ovulation in albino rats of Wistar strain. Niger Postgrad Med J 2006;13:203–5.
[27] Gu Y, Lin YC, Rikihisa Y. Inhibitory effect of gossypol on steroidogenic pathways in cultured bovine luteal cells. Biochem Biophys Res Commun 1990;169:455–61.
[28] Moh PP, Chang GC, Brueggemeier RW, Lin YC. Effect of gossypol on 5 alpha-reductase and 3 alpha-hydroxysteroid dehydrogenase activities in adult rat testes. Res Commun Chem Pathol Pharmacol 1993;82:12–26.
[29] Moh PP, Chang GC, Hu YF, Brueggemeier RW, Lin YC. Effect of gossypol on 5 alpha-reductase activity in canine benign prostatic hyperplasia. FASEB J 1992;7A:237.
[30] Basini G, Baratta M, Ponderato N, Bussolati S, Tamanini C. Is nitric oxide an autocrine modulator of bovine granulosa cell function? Reprod Fertil Dev 1998;10:471–8.
[31] Grasselli F, Ponderato N, Basini G, Tamanini C. Nitric oxide synthase expression and nitric oxide/cyclic GMP pathway in swine granulosa cells. Domest Anim Endocrinol 2001;20:241–52.
[32] Vranová J, Jezová M, Scsuková S, Kolena J. Inhibitory effect of gossypol on basal and luteinization factor-stimulated progesterone synthesis in porcine granulosa cells. Physiol Res 1999;48:119–28.
[33] Masuda M, Kubota T, Aso T. Effects of nitric oxide on steroidogenesis in porcine granulosa cells during different stages of follicular development. Eur J Endocrinol 2001;144:303–8.
[34] Kostic TS, Andric SA, Maric D, Kovacevic RZ. Inhibitory effects of stress-activated nitric oxide on antioxidant enzymes and testicular steroidogenesis. J Steroid Biochem Mol Biol 2000;75:299–306.
[35] Faes MR, Caldas-Bussiere MC, Viana KS, Dias BL, Costa FR, Escocard RM. Nitric oxide regulates steroid synthesis by bovine antral granulosa cells in a chemically defined medium. Anim Reprod Sci 2008. Feb 7, [Epub ahead of print].
[36] Basini G, Grasselli F, Bianco F, Tirelli M, Tamanini C. Effect of reduced oxygen tension on reactive oxygen species production and activity of antioxidant enzymes in swine granulosa cells. Biofactors 2004;20:61–9.
[37] Basini G, Bussolati S, Santini SE, Grasselli F. Reactive oxygen species and anti-oxidant defences in swine follicular fluids. Reprod Fertil Dev 2008;20:269–74.
[38] Kovacic P. Mechanism of drug and toxic actions of gossypol: focus on reactive oxygen species and electron transfer. Curr Med Chem 2003;10:2711–8.
[39] Pellegrini N, Serafini M, Colombi B, Del Rio D, Salvatore S, Bianchi M, Brighenti F. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. J Nutr 2003;133:2812–9.
[40] Wang X, Beckham TH, Morris JC, Chen F, Gangemi JD. Bioactivities of gossypol, 6-methoxygossypol, and 6,6'-dimethoxygossypol. J Agric Food Chem 2008;56:4393–8.
[41] Xu L, Yang D, Wang S, Tang W, Liu M, Davis M, et al. (−)-Gossypol enhances response to radiation therapy and results in tumor regression of human prostate cancer. Mol Cancer Ther 2005;4:197–205.
[42] Meng Y, Tang W, Dai Y, Wu X, Liu M, Ji Q, Ji M, Pienta K, Lawrence T, Xu L. Natural BH3 mimetic (−)-gossypol chemosensitizes human prostate cancer via Bcl-xL inhibition accompanied by increase of Puma and Noxa. Mol Cancer Ther 2008;7:2192–202.
[43] Hu ZY, Zhu XF, Zhong ZD, Sun J, Wang J, Yang D, Zeng YX. ApoG2, a novel inhibitor of antiapoptotic Bcl-2 family proteins, induces apoptosis and suppresses tumor growth in nasopharyngeal carcinoma xenografts. Int J Cancer 2008;123:2418–29.
[44] Etxebarria A, Landeta O, Antonsson B, Basañez G. Regulation of antiapoptotic MCL-1 function by gossypol: mechanistic insights from in vitro reconstituted systems. Biochem Pharmacol 2008;76:1563–76.
[45] Sun Y, Wu J, Aboukameel A, Banerjee S, Arnold AA, Chen J, Nikolovska-Coleska Z, Lin Y, Ling X, Yang D, Wang S, Al-Katib A, Mohammad RM. Apogossypolone, a nonpeptidic small molecule inhibitor targeting Bcl-2 family proteins, effectively inhibits growth of diffuse large cell lymphoma cells in vitro and in vivo. Cancer Biol Ther 2008;7:1418–26.

UNCORRECTED PROOF

Please cite this article in press as: Basini G, et al. Gossypol, a polyphenolic aldehyde from cotton plant, interferes with swine granulosa cell function. Domest Anim Endocrinol (2009), doi:10.1016/j.domaniend.2009.01.005

ALLEGATO G

"Bisphenol A disrupts granulosa cell function".

Domestic Animal Endocrinology. 2010. 39: 34-39.
doi:10.1016/j.domaniend.2010.01.004

ELSEVIER
Available online at www.sciencedirect.com
ScienceDirect
Domestic Animal Endocrinology xxx (2010) xxx–xxx
DOMESTIC ANIMAL ENDOCRINOLOGY
www.domesticanimalendo.com

Short communication

1 # Bisphenol A disrupts granulosa cell function

2 F. Grasselli [a], L. Baratta [a], L. Baioni [a], S. Bussolati [a],
3 R. Ramoni [b], S. Grolli [b], G. Basini [a,*]

4 Q1 [a] Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti, Italy
5 [b] Sezione di Fisiologia and Sezione di Biochimica, Università degli Studi di Parma, Italy

Received 13 October 2009; received in revised form 8 January 2010; accepted 10 January 2010

6

7 **Abstract**

8 Because of its widespread use and potential adverse biological effects, bisphenol A (BPA) represents one of the most studied
9 endocrine-disrupting compounds. Within the reproductive system, ovarian granulosa cells have been documented as a target of
10 BPA action, but no consensus has been reached about functional modifications induced by BPA. On these bases, we studied the
11 potential disrupting effects of BPA on the main granulosa cell functional activities, also taking into account a potential interference
12 with the ovarian angiogenic process. Ovarian granulosa cells were isolated from porcine follicles and cultured in the presence or
13 absence of BPA at different concentrations for 48 h. Cell proliferation was studied by measuring adenosine triphosphate content.
14 Progesterone (P4) and estradiol 17β (E2) production was determined by radioimmunoassay. Vascular endothelial growth factor
15 (VEGF) output was quantified by an enzyme-linked immunosorbent assay. Redox status was monitored by measuring superoxide
16 anion and hydrogen peroxide, and by determining the activities of the scavenging enzymes superoxide dismutase, catalase, and
17 peroxidase by colorimetric methods. Granulosa cell proliferation as well as redox status resulted unaffected by BPA. Concentrations
18 of E2 were stimulated by the lower BPA concentration, whereas they were inhibited by the larger doses tested. P4 output was
19 decreased by all BPA concentrations. To the contrary, VEGF production was stimulated. Data indicate that BPA can interfere with
20 reproductive activity by affecting granulosa cell steroidogenesis in vitro; furthermore, BPA can exert a promoting effect on the
21 ovarian angiogenic process by increasing VEGF output in pigs. A disruption of this finely tuned process seems particularly relevant
22 because of the risk of uncontrolled neovascularization.
23 © 2010 Published by Elsevier Inc.

24 *Keywords:* Endocrine disruptors; Granulosa cell; Steroidogenesis; Angiogenesis; Redox status

25

26 ## 1. Introduction

27 A normally functioning endocrine system must be
28 in place for normal follicle development and genera-
29 tion of good-quality oocytes. Therefore, it is important
30 to take into account rising concerns about possi-
31 ble adverse effects of environmental contaminants,
32 especially endocrine disruptors. Endocrine-disrupting
33 compounds include naturally-occurring substances, as
34 well as synthetic chemicals, that can interfere with the
35 endocrine axis of vertebrates [1,2]. A specific group of
36 compounds termed plasticizers have been proposed to
37 alter endocrine function [3].

38 Bisphenol A (BPA), used as plasticizer in the man-
39 ufacture of polycarbonate plastics and epoxy resins, is
40 present in a multitude of products, including the interior
41 coatings of food cans, milk containers, and baby for-
42 mula bottles, as well as in dental sealants. Studies have
43 shown that depolymerization may occur causing BPA
44 to leach into foods [4], infant formula [5] or saliva [6].

* Corresponding author at: Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti, Sezione di Fisiologia Veterinaria, Via del Taglio 10, 43126 Parma, Italy. Tel.: +39 0521 032775; fax: +39 0521 032770.
E-mail address: basini@unipr.it (G. Basini).

0739-7240/$ – see front matter © 2010 Published by Elsevier Inc.
doi:10.1016/j.domaniend.2010.01.004

UNCORRECTED PROOF

The dramatically increasing human exposure to BPA is evidenced by a detection of BPA in human serum and follicular fluid, as well as in amniotic fluid and fetal serum [7]. These findings have generated both scientific and public interest in assessing potential health risks. In particular, morphological and functional modifications after exposure to BPA have been documented in the ovary [8].

Granulosa cells are essential for ovarian follicle growth, steroidogenesis and oocyte survival and nourishment. In addition, they produce vascular endothelial growth factor (VEGF). This peptide has a pivotal role in the physiological angiogenesis that occurs cyclically in the ovary, driving the normal development and growth of ovarian follicles [9]. The expression of VEGF has been documented to be induced by BPA in several reproductive tissues such as rat uterus and vagina [10], but its effects on VEGF output by granulosa cells has not been reported.

On these bases, the current in vitro study was undertaken to evaluate the potential disrupting effects of BPA on two main granulosa cell functional activities, proliferation and steroidogenesis. Moreover, we also evaluated the impact of BPA on the ovarian angiogenic process by verifying its action on granulosa cell VEGF production. Since we have previously documented a signalling role of reactive oxygen species in the angiogenic cascade triggered by VEGF [11], we also focused our attention on cell redox status after treatment with BPA.

2. Materials and methods

All reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

2.1. Granulosa cell culture

Swine ovaries were collected at a local slaughterhouse, placed into cold PBS (4 °C) supplemented with penicillin (500 IU/mL), streptomycin (500 μg/mL) and amphotericin B (3.75 μg/mL), maintained in a freezer bag and transported to the laboratory within 1 h. After 2 washings with PBS and ethanol (70%), granulosa cells were aseptically harvested by aspiration from follicles > 5 mm with a 26-gauge needle and released in medium containing heparin (50 IU/mL), centrifuged for pelleting, and treated with 0.9% prewarmed ammonium chloride at 37 °C for 1 min to remove red blood cells. Cell number and viability were estimated using a haemocytometer under a phase contrast microscope after vital staining with trypan blue (0.4%) of an aliquot of cell suspension. To optimize analytical performance [12–14], cells were seeded at different densities (see below) in culture medium (CM) consisting of DMEM Ham's F12 supplemented with sodium bicarbonate (2.2 mg/mL), bovine serum albumin (0.1%), penicillin (100 UI/mL), streptomycin (100 μg/mL), amphotericin B (2.5 μg/mL), selenium (5ng/mL), and transferrin (5 μg/mL). Cells were incubated with BPA at concentrations of 0, 0.1, 1 or 10 μM and incubated at 37 °C under a humidified atmosphere (5% CO_2) for 48 h. These concentrations were chosen on the basis of previously reported studies [15,16]. Bisphenol A was initially dissolved in dimethyl sulfoxide (DMSO) and at the time of use it was diluted to the required concentrations with culture medium. The final DMSO concentration was added to the control group.

2.1.1. Granulosa cell proliferation

Cell viability was assayed using a bioluminescent assay (ATP-lites; Packard Bioscience, Groningen, Netherlands) which measured intracellular ATP levels as an indicator of cell numbers. ATP is a cell viability marker because it is present in all metabolically-active cells, and the concentration declines very rapidly when the cells undergo necrosis or apoptosis. The ATP lite-M assay system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. This is illustrated in the following reaction scheme: ATP + D-luciferin+ O_2 → luciferase, Mg^{2+}→ oxyluciferin + AMP + PPi + CO_2 + light. The emitted light is proportional to the ATP concentration. In order to assess proliferation, 2×10^5 cells were seeded in 96-well plates in 200 μL CM. At the end of 48 h incubation with BPA at the above mentioned concentrations, 50 μL of mammalian cell lysis solution were added to 100 μL of cell suspension and the plate was shaken for 5 min in an orbital shaker at 700 rpm in order to lyse the cells and stabilize ATP. Fifty μL of substrate solution was added to the wells and the microplate was shaken for 5 min in an orbital shaker at 700 rpm. The plate was placed in the dark for 10 min and the luminescence was measured in a luminometer (Victor, Packard Bioscience, Groningen, Netherlands).

2.1.2. Granulosa cell steroid production

Cells (10^4 cells/well) were seeded in 96-well plates in 200 μL CM supplemented with androstenedione (28 ng/mL). Culture medium was collected after incubation, frozen and stored at -20 °C until progesterone (P4) and estradiol-17β (E2) determination by validated RIA [17]. Sensitivities and ED50 for P4 and E2 assays were 75 and 314 pg/mL, and 13.2 and 54.5 pg/mL, respectively. The intra- and inter-assay coefficients of variation were less than 12% for both assays.

UNCORRECTED PROOF

Please cite this article in press as: Grasselli F, et al. Bisphenol A disrupts granulosa cell function. Domest Anim Endocrinol (2010), doi:10.1016/j.domaniend.2010.01.004

2.1.3. Granulosa cell VEGF production

The VEGF concentration in the culture media was quantified by an ELISA (Quantikine, R&D System, Minneapolis, MI, USA). This assay, which was developed for human VEGF detection, has been validated for pig VEGF [18]. Briefly, 10^6 cells/mL CM were seeded in 24-well plates and the assay was performed on culture media after 48 h of incubation at 37 °C under a humidified atmosphere (5% CO_2). The assay sensitivity was 8.74 pg/mL, and the intra and inter-assay CVs were always less than 7%. The absorbance was determined with a Victor Reader using a 450 nm filter.

2.1.4. Granulosa cell reactive oxygen species production

Superoxide anion (O_2^-) generation was measured by the cell proliferation WST-1 test (Roche, Mannheim, Germany). Briefly, 10^5 cells/200 μL CM were seeded in 96-well plates. Since evidence exists [19,20] that tetrazolium salts can be used as a reliable measure of intracellular O_2^- production, 20 μL of WST-1 were added to the cell during the last 4 h of incubation. The absorbance was then determined using a Victor Reader at 450 nm against 620 nm.

Hydrogen peroxide (H_2O_2) production was measured by an Amplex Red hydrogen peroxide Assay Kit (Molecular Probes, PoortGebouw, The Netherlands); the Amplex Red reagent reacts with H_2O_2 to produce resorufin, an oxidation product. Briefly, $2x10^5$ cells/200 μL CM were seeded in 96-well plates. After incubation with treatments, plates were centrifuged for 10 min at 400 × g, the supernatants were discarded, and cells were lysed by adding cold Triton 1% in Tris–HCl (100 μL per well), and incubating on ice for 30 min. The test was performed on cell lysates and read against a standard curve of H_2O_2 ranging from 0.195 to 12.5 μM. A Victor microplate reader set to read 540 nm emission was used to quantify the reaction product.

2.1.5. Granulosa cell scavenger enzyme activities

Cells were seeded in 96-well plates (2×10^5 /200 μl CM) and incubated for 48 h. After centrifugation for 10 min at 400 × g, supernatants were discarded and cells were lysed adding cold Triton 1% in TRIS HCl (100 μL/10^5 cells) and incubated on ice for 30 min. Superoxide dismutase (SOD), catalase and peroxidase activities were assessed in cell lysates as described below. Superoxide dismutase activity was determined by a SOD Assay Kit (Dojindo Molecular Technologies, Japan). Cell lysates were tested without dilution and a standard curve ranging from 0.156 to 20 U/mL was prepared. The colorimetric assay was performed measuring formazan produced by the reaction between tetrazolium salt (WST-1) and superoxide anion (O_2^-), produced by the reaction of an exogenous xantine oxidase. The remaining O_2^- is an indirect marker for the endogenous SOD activity. The absorbance was determined with a Victor Reader reading at 450 nm against 620 nm.

Catalase activity was measured by the Amplex Red catalase assay Kit (Molecular Probes) based on the formation of an oxidation product (resorufin) derived from the reaction between H_2O_2 given in excess, and the Amplex Red reagent in the presence of horseradish peroxidase. Cell lysates were diluted 1:10 to perform the test and read against a standard curve of catalase ranging from 62.5 to 1000 mU/mL. The absorbance was determined with Victor Reader using a 540 nm filter.

Peroxidase activity was measured by the Amplex Red peroxidase assay kit (Molecular Probes) based on the formation of an oxidation product (resorufin) derived from the reaction beetwen H_2O_2 given in excess and the Amplex Red reagent. Cell lysates were used undiluted to perform the test and read against a standard curve of peroxidase ranging from 0.039 to 5 mU/mL. The absorbance was determined with Victor Reader using a 540 nm filter.

2.2. Statistical analyses

Each experiment was repeated at least 4 times with 6 replicates for each treatment (n = 24). Experimental data are presented as mean ± SEM; statistical differences were calculated with ANOVA using the Statgraphics package (STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by Scheffè's F test.

3. Results

3.1. Granulosa cell proliferation, and steroid and VEGF production

Swine granulosa cell proliferation was evaluated as mean (± S.E.M) ATP content (basal = 16.5 ± 0.9 μM) and was unaffected by different concentrations of BPA. Basal steroid production by granulosa cells was 3.7 ± 0.1 and 71.8 ± 3 ng/mL for E2 and P4, respectively. Addition of 0.1 μM BPA stimulated ($P < 0.001$) E2 production in granulosa cells, whereas 1 and 10 μM ($P < 0.001$) displayed inhibitory effects (Fig. 1 A). Production of P4 by granulosa cells was inhibited ($P < 0.01$) by BPA at all concentrations tested (Fig. 1 B). Release of VEGF by swine granulosa cells was unaffected by the lowest concentration of BPA but was stimulated ($P < 0.05$) by 1 and 10 μM BPA (Fig. 1 C).

Please cite this article in press as: Grasselli F, et al. Bisphenol A disrupts granulosa cell function. Domest Anim Endocrinol (2010), doi:10.1016/j.domaniend.2010.01.004

UNCORRECTED PROOF

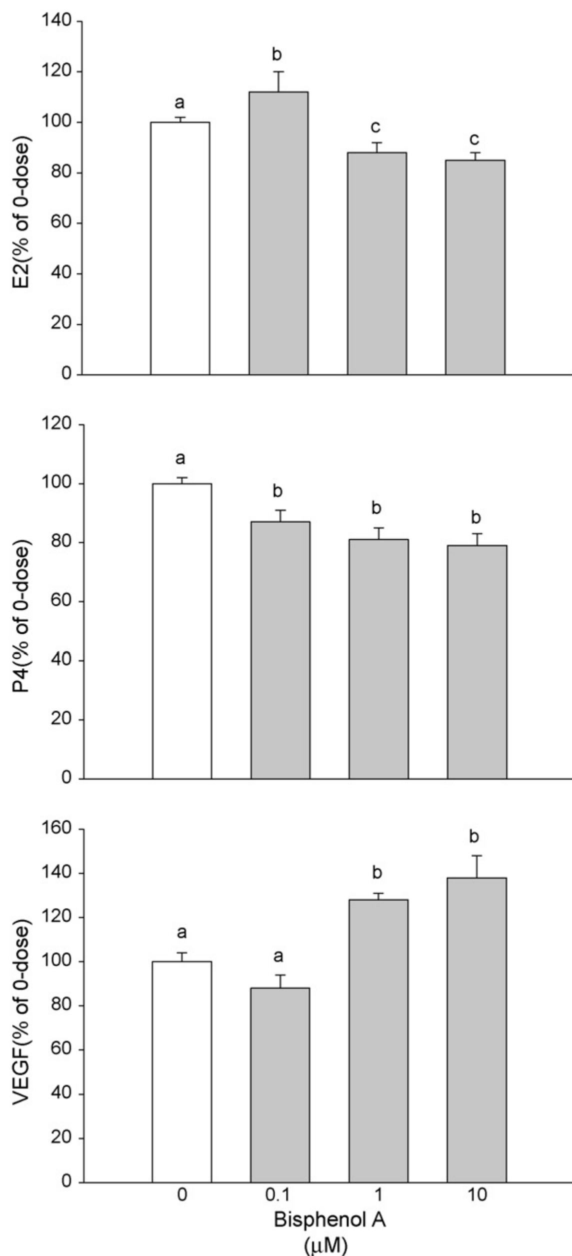


Fig. 1. Effect of 48-h treatment with or without Bisphenol A at concentrations of 0, 0.1, 1, and 10 μM on granulosa cell E2 (A), P4 (B), and VEGF secretion (C). Data represent mean (± SEM) concentrations of hormones expressed as percent of controls (0 dose) in media of six replicates/treatment repeated in 4 different experiments (n = 24). Different letters indicate a ignificant difference ($P < 0.05$).

3.2. Granulosa cell reactive oxygen species production and scavenger enzyme activities

Treatment with BPA was ineffective in modulating superoxide anion (basal value = 380 ± 15 mass units), hydrogen peroxide (basal value = 1.7 ± 0.1 μM), SOD (basal value = 320 ± 10 mU/mL), catalase (basal value = 32 ± 7 mU/mL) and peroxidase (basal value = 1420 ± 220 mU/mL) activities in cultured swine granulosa cells.

4. Discussion

The widespread distribution and environmental persistence of the xenoestrogen, BPA likely results in a high potential for human and animal exposure. This has recently created public concern and many studies have focused on the potential of BPA to cause adverse effects on reproduction and development [21]. In particular, BPA exposure has been associated with recurrent miscarriage in women [22] and serum levels of BPA were demonstrated to be greater in women with polycystic ovarian syndrome (PCOS) [23] compared to healthy controls. Several studies in mammals and fish have provided documentation that BPA affects both morphology and function of reproductive organs [24–26]. However, doubts concerning the validity of this viewpoint have arisen recently [27] and, according to an expert panel convened by the National Toxicology Program (USA, 2008; http://cerhr.niehs.nih.gov/chemicals/bisphenol/bisphenol.pdf) it was concluded that reproductive effects due to BPA exposure are negligible [8].

It is well known that granulosa cells play a crucial role in ovarian physiology through the production of steroid hormones, as well as other factors that interact with the oocyte during its development [28]. Hence, a disruption of their functional activities by BPA could have a significant impact on reproductive efficiency.

Our data do not substantiate a cytotoxic action of BPA, nor a significant effect on cell redox status in our experimental model. These results are in contrast with previous experimental evidence. In particular, Yang et al. [29] demonstrated that BPA exposure is associated with oxidative stress in postmenopausal women, while Xu et al. [30] documented BPA inhibitory effects on mouse granulosa cell proliferation. These discrepancies may be attributable first of all to species differences; moreover, lower BPA concentrations (in the μM range) were tested in our cell culture, and only the effects of a short-term cell exposure (48 h) to BPA were examined.

Contrasting results have also been reported regarding the action of BPA on granulosa cell steroid production. A significant inhibitory action on estradiol production was reported in swine [31], whereas no effects on steroidogenic pathways were observed in the mouse [32]. The current in vitro study indicates that BPA can disrupt granulosa cell function by interfering with steroidogenesis. In particular, E2 production was inhibited by 1

UNCORRECTED PROOF

Please cite this article in press as: Grasselli F, et al. Bisphenol A disrupts granulosa cell function. Domest Anim Endocrinol (2010), doi:10.1016/j.domaniend.2010.01.004

and 10 μM BPA, which is in accordance with the observations of Zhou et al. [16], who reported a decrease in concentrations of E2 in the rat at similar BPA concentrations, as well as a decrease in mRNA expression of P450 aromatase, a key enzyme for estradiol production. A stimulatory effect of BPA was observed on E2 secretion at the lowest BPA concentration tested in our study. We interpret this as a hormonal response of granulosa cells, which is observed frequently in response to estrogenic endocrine-disrupting chemicals [33]. To the contrary, BPA inhibited P4 production at all concentrations tested. A suppressive effect of BPA on basal P4 production by rat granulosa cells has been documented recently by Zhou et al. [16], supporting the hypothesis that BPA has negative effects on the expression of P450 side-chain cleavage enzyme mRNA. Overall, our data confirm that BPA disrupts ovarian steroidogenesis. However, further studies will be needed in order to verify a potential effect on the expression of ovarian steroidogenic enzymes.

The current work also demonstrated that both BPA addition between 1 and 10 μM significantly stimulates VEGF secretion in swine granulosa cells. An induction of VEGF expression by BPA has been reported previously in breast cancer cells [34], but to the best of our knowledge, this is the first report of a modulating action of BPA on VEGF output within the ovary. This result is of particular interest, given that VEGF plays a fundamental role in the angiogenic process that promotes endothelial cell growth and permeability [35]. This process triggers neovascularization in both normal adult and in neoplastic tissue. The ovarian follicle represents a unique example of physiological angiogenesis since the development of an extensive vascular network is strictly linked with its growth and final maturation to ovulation. On the other hand, growing antral follicles also represent valuable models to study pathological angiogenesis because of their close similarity to solid tumours, highly vascularized structures whose progression also heavily depend on neovascularization [36]. Therefore, in accordance with data obtained in other reproductive tissues [37], our observations indicate that endocrine-disrupting effects of BPA on reproductive activity can be associated with a marked promotion of vascular permeability, a process that contributes to the timing of antrum formation. Moreover, BPA can induce angiogenesis which physiologically represents a finely-tuned process resulting from a delicate balance between pro- and anti-angiogenic agents. The stimulatory effects of BPA on VEGF production may have negative implications, potentially switching the balance toward uncontrolled neovascularization and thus the development of pathological processes. This hypothesis needs to be substantiated by future studies addressing this issue.

In conclusion, the current study demonstrated that BPA affects granulosa cell steroidogenesis in vitro and increases VEGF production. A disruption of these finely-tuned events could interfere with reproductive efficiency.

Acknowledgements

This study was supprted by a CARIPARMA grant .

References

[1] Kang JH, Kondo F, Katayama Y. Human exposure to bisphenol A. Toxicology 2006;226:79–89.
[2] Sekizawa J. Low-dose effects of bisphenol-A: a serious threat to human health? J Toxicol Sci 2008;33:389–403.
[3] Oehlmann J, Oetken M, Schulte-Oehlmann U. A critical evaluation of the environmental risk assessment for plasticizers in the freshwater environment in Europe, with special emphasis on bisphenol A and endocrine disruption. Environ Res 2008;108:140–9.
[4] Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N. Xenoestrogens released from lacquer coatings in food cans. Environ Health Perspect 1995;103:608–12.
[5] Biles JE, White KD, McNeal TP, Begley TH. Determination of the diglycidyl ether of bisphenol A and its derivatives in canned foods. J Agric Food Chem 1999;47:1965–9.
[6] Olea N, Pulgar R, Pérez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V, Soto AM, Sonnenschein C. Estrogenicity of resin-based composites and sealants used in dentistry. Environ Health Perspect 1996;104:298–305.
[7] Ikezuki Y, Tsutsumi O, Takai Y, Kamei Y, Taketani Y. Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. Hum Reprod 2002;17:2839–41.
[8] Hunt PA, Susiarjo M, Rubio C, Hassold TJ. The bisphenol A experience: a primer for the analysis of environmental effects on mammalian reprodution. Biol Reprod 2009 [Epub ahead of print].
[9] Basini G, Bussolati S, Santini SE, Bianchi F, Careri M, Mangia A, et al. Hydroxyestrogens inhibit angiogenesis in swine ovarian follicles. J Endocrinol 2008;199:127–35.
[10] Long X, Steinmetz R, Ben-Jonathan N, Caperell-Grant A, Young PC, Nephew KP, et al. Strain differences in vaginal responses to the xenoestrogen bisphenol A. Environ Health Perspect 2000;108:243–7.
[11] Basini G, Bussolati S, Baioni L, Grasselli F. Gossypol, a polyphenolic aldehyde from cotton plant, interferes with swine granulosa cell function. Domest Anim Endocrinol 2009;37:30–6.
[12] Basini G, Tamanini C. Selenium stimulates estradiol production in bovine granulosa cells: possible involvement of nitric oxide. Domest Anim Endocrinol 2000;18:1–17.
[13] Basini G, Bianco F, Grasselli F, Tirelli M, Bussolati S, Tamanini C. The effects of reduced oxygen tension on swine granulosa cell. Regul Pept 2004;120:69–75.
[14] Basini G, Grasselli F, Bianco F, Tirelli M, Tamanini C. Effect of reduced oxygen tension on reactive oxygen species production and activity of antioxidant enzymes in swine granulosa cells. Biofactors 2004;20:61–9.

UNCORRECTED PROOF

[15] Dominguez MA, Petre MA, Neal MS, Foster WG, Bisphenol. A concentration-dependently increases human granulosa-lutein cell matrix metalloproteinase-9 (MMP-9) enzyme output. Reprod Toxicol 2008;25:420–5.
[16] Zhou W, Liu J, Liao L, Han S, Liu J. Effect of bisphenol A on steroid hormone production in rat ovarian theca-interstitial and granulosa cells. Mol Cell Endocrinol 2008;283:12–8.
[17] Grasselli F, Baratta M, Tamanini C. Effects of a GnRH analogue (buserelin) infused via osmotic minipumps on pituitary and ovarian activity of prepuberal heifers. Anim Reprod Sci 1993;32:153–61.
[18] Barboni B, Turriani M, Galeati G, Spinaci M, Bacci ML, Forni M, et al. Vascular endothelial growth factor production in growing pig antral follicles. Biol Reprod 2000;63:858–64.
[19] Benov L, Fridovich I. Is reduction of the sulfonated tetrazolium 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2-tetrazolium 5-carboxanilide a reliable measure of intracellular superoxide production? Anal Biochem 2002;310:186–90.
[20] Ukeda H, Shimamura T, Tsubouchi M, Harada Y, Nakai Y, Sawamura M. Spectrophotometric assay of superoxide anion formed in Maillard reaction based on highly water-soluble tetrazolium salt. Anal Sci 2002;18:1151–4.
[21] Maffini MV, Rubin BS, Sonnenschein C, Soto AM. Endocrine disruptors and reproductive health: the case of bisphenol-A. Mol Cell Endocrinol 2006;254-5:179–86.
[22] Sugiura-Ogasawara M, Ozaki Y, Sonta S, Makino T, Suzumori K. Exposure to bisphenol A is associated with recurrent miscarriage. Hum Reprod 2005;20:2325–9.
[23] Takeuchi T, Tsutsumi O, Ikezuki Y, Takai Y, Taketani Y. Positive relationship between androgen and the endocrine disruptor, bisphenol A, in normal women and women with ovarian dysfunction. Endocr J 2004;51:165–9.
[24] Berger RG, Shaw J, deCatanzaro D. Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone and 17 beta-estradiol. Reprod Toxicol 2008;26:94–9.
[25] Mandich A, Bottero S, Benfenati E, Cevasco A, Erratico C, Maggioni S, et al. In vivo exposure of carp to graded concentrations of bisphenol A. Gen Comp Endocrinol 2007;53:15–24.
[26] Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, et al. In vitro molecular mechanisms of bisphenol A action. Reprod Toxicol 2007;24:178–98.
[27] Goodman JE, Witorsch RJ, McConnell EE, Sipes IG, Slayton TM, Yu CJ, et al. Weight-of-evidence evaluation of reproductive and developmental effects of low doses of bisphenol A. Crit Rev Toxicol 2009;39:1–75.
[28] Su YQ, Sugiura K, Eppig JJ. Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism. Semin Reprod Med 2009;27:32–42.
[29] Yang YJ, Hong YC, Oh SY, Park MS, Kim H, Leem JH. Et al., Bisphenol A exposure is associated with oxidative stress and inflammation in postmenopausal women. Environ Res. 2009.[Epub ahead of print].
[30] Xu J, Osuga Y, Yano T, Morita Y, Tang X, Fujiwara T, et al. Bisphenol A induces apoptosis and G2-to-M arrest of ovarian granulosa cells. Biochem Biophys Res Commun 2002;292:456–62.
[31] Mlynarcíková A, Kolena J, Ficková M, Scsuková S. Alterations in steroid hormone production by porcine ovarian granulosa cells caused by bisphenol A and bisphenol A dimethacrylate. Mol Cell Endocrinol 2005;244:57–62.
[32] Lenie S, Cortvrindt R, Eichenlaub-Ritter U, Smitz J. Continuous exposure to bisphenol A during in vitro follicular development induces meiotic abnormalities. Mutat Res 2008;651:71–81.
[33] Calabrese EJ. Hormesis: why it is important to toxicology and toxicologists. Environ Toxicol Chem 2008;27:1451–74.
[34] Buteau-Lozano H, Velasco G, Cristofari M, Balaguer P, Perrot-Applanat M. Xenoestrogens modulate vascular endothelial growth factor secretion in breast cancer cells through an estrogen receptor-dependent mechanism. J Endocrinol 2008;96:399–412.
[35] Kaczmarek MM, Schams D, Ziecik AJ. Role of vascular endothelial growth factor in ovarian physiology - an overview. Reprod Biol 2005;5:111–36.
[36] Neeman M, Abramovitch R, Schiffenbauer YS, Tempel C. Regulation of angiogenesis by hypoxic stress: from solid tumours to the ovarian follicle. Int J Exp Pathol 1997;78:57–70.
[37] Bredhult C, Bäcklin BM, Olovsson M. Effects of some endocrine disruptors on the proliferation and viability of human endometrial endothelial cells in vitro. Reprod Toxicol 2007;23:550–9.

UNCORRECTED PROOF

Please cite this article in press as: Grasselli F, et al. Bisphenol A disrupts granulosa cell function. Domest Anim Endocrinol (2010), doi:10.1016/j.domaniend.2010.01.004

ALLEGATO H

"Antiangiogenic Resveratrol Analogues by Mild m-CPBA Aromatic Hydroxylation of 3,5-Dimethoxystilbenes"

Natural Product Communication. 2009. 4: 239-246.

NPC Natural Product Communications

2008
Vol. 3
No. 0
1 - 9

Antiangiogenic Resveratrol Analogues by Mild *m*-CPBA Aromatic Hydroxylation of 3,5-Dimethoxystilbenes

Carmela Spatafora,[a] Giuseppina Basini,[b] Laura Baioni,[b] Francesca Grasselli,[b] Alfio Sofia[a] and Corrado Tringali[a]*

[a]*Dipartimento di Scienze Chimiche*, *Università di Catania*, *Viale A. Doria 6*, *I-95125 Catania*, *Italy*

[b]*Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti, Università di Parma, Via del Taglio 8, I-43100 Parma, Italy*

ctringali@unict.it

Received: November10th, 2008; Accepted: XX, 2008

A mild treatment of the resveratrol analogue 3,5,4'-trimethoxystilbene **2** with *m*-CPBA afforded two hydroxylated methoxystilbenes (**5** and **6**) by direct aromatic hydroxylation. A similar protocol was applied to other stilbenes bearing a 3,5-dimethoxy moiety, namely the tetramethoxystilbenes **7** and **10** to obtain respectively the hydroxylated analogues **8**, **9** and **11**, **12**. The substrate **2** and the new compounds **5**, **8** and **11** were evaluated as antiangiogenic agents employing a method previously developed by some of us, and proved significantly active in the range 1 – 100 µM.

Keywords: antiangiogenic activity, resveratrol analogues, methoxystilbenes, hydroxylation, *m*-CPBA.

E-resveratrol (*E*-3,5,4′-trihydroxystilbene, **1**, Figure 1), originally isolated from *Veratrum grandiflorum* [1] and later obtained from *Polygonum cuspidatum* [2], is one of the main polyphenols present in red wine. It has became popular mainly due to the so-called 'French paradox', namely the inverse correlation between a high-fat diet and low mortality risk of heart disease, observed in some French Southern regions and attributed to red wine consumption [3]. Starting from the first studies focused on its possible role in preventing cardiovascular heart diseases (CHD) [4], as well as on its cancer chemopreventive properties [5], this stilbenoid has recently attracted the attention of an increasing number of researchers. In particular, *E*-resveratrol exhibits promising antiproliferative, pro-apoptotic and antiangiogenic properties [6-10]. Nevertheless, the available *in vivo* studies indicate that **1**, although absorbed in high extent by the organism, has a poor bioavailability [11], and may be converted *in vivo* into compounds lacking of its anti-proliferative activity [9]. Thus, a number of resveratrol analogues have been recently evaluated as anti-tumor agents, in the hope to obtain compounds with higher activity and / or bioavailability. Among these, polymethoxystilbenes are an interesting sub-group including compounds with significant antitumor properties [12]. In particular, the presence of a 3,5-dimethoxy moiety is frequently associated with noticeable biological activity. In addition, some *in vivo* studies indicate that methoxystilbenes undergo different metabolic conversion and have a higher bioavailability with respect to resveratrol [13]. In recent studies the simple *E*-resveratrol derivative 3,5,4'-trimethoxystilbene **2** (Figure 1), and its *Z*-isomer (**3**, Figure 1), structurally related to the potent tubulin inhibitor and vascular disrupting agent combretastatin A4 (**4**, Figure 1) [14], proved far more active than **1** both as antiproliferative [13] and antiangiogenic agents [15].

1 R = H

2 R = CH_3

3 $R_1 = R_2 = H$

4 $R_1 = OCH_3$, $R_2 = OH$

Figure 1: Structures of compounds **1 - 4**

In particular, **2** was 30 to 100 times more potent than **1** in inhibiting endothelial cell proliferation, sprouting, collagen gel invasion, and morphogenesis. *In vivo*, **2** caused the rapid stasis of blood flow and regression of intersegmental vessels in the trunk of zebrafish embryos. In addition, it inhibited blood vessel growth and caused the disappearance of pre-existing blood vessels in the chick embryo.

A rising interest towards new vessel growth reducing agents has been observed in the last decade, due to the possible applications of these substances in cancer therapy, as well as in other degenerative diseases involving new vessel growth. Unfortunately, while the major stimula triggering the neovascularization process are overall well characterized, the mechanisms underlying the negative control of angiogenesis are still not completely known. In previous study, several of us attempted to unravel physiological mechanisms of neovascularisation [16-20] focusing, more recently, on its negative control [21-23]. Various angiogenesis inhibitors have been developed so far; their efficacy has been evaluated in different in vitro and in vivo assays, and their clinical evaluation is in progress [24]. Recently, the anti-VEGF antibody bevacizumab has been shown to exert antiangiogenic effects in patients with cancer, leading to U.S. Food and Drug Administration approval for colorectal cancer treatment [25].

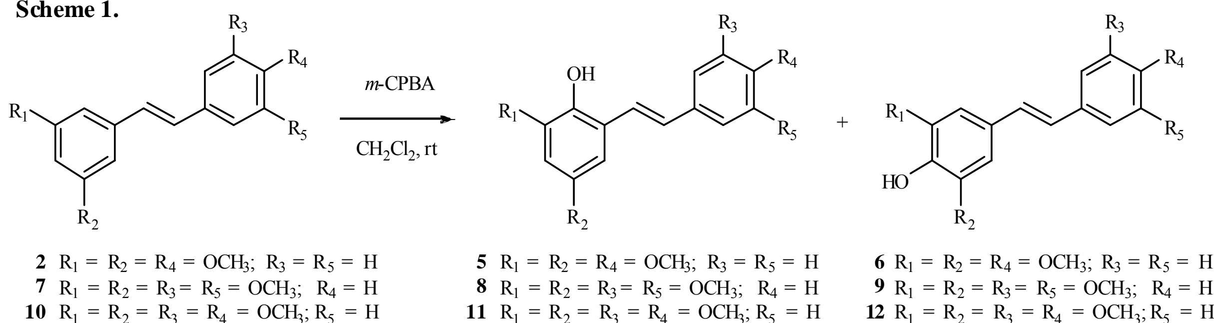
These observations prompted us, in continuation of our studies on resveratrol analogues [26, 27], to synthesize further methoxystilbenes as new antiangiogenic agents.

As detailed in the following sections, we employed a mild treatment of substrate **2** with *m*-CPBA at rt to obtain two hydroxylated methoxystilbenes (**5** and **6**). Analogously, a similar protocol was applied to the tetramethoxystilbenes **7** and **10** to obtain respectively the hydroxylated analogues **8**, **9** and **11**, **12**. Among these resveratrol analogues, we selected the substrate **2** and the new compounds **5**, **8** and **11** for an evaluation of their angiogenic properties employing our previously developed method.

In contrast with the variety of completely methoxylated stilbenoids prepared by standard synthetic methods [13], methoxystilbenes bearing also one free phenol group have been reported rarely, and obtained by cumbersome synthetic methodologies, normally affording a mixture of *cis* and *trans* isomers [28, 29]. This is probably the reason for the scarcity of literature data about the biological properties of hydroxylated polimethoxystilbenes. In searching for simple methods to obtain this kind of resveratrol analogues, we were attracted by the unexpected result of a standard treatment of substrate **2** with *m*-CPBA, carried out as a side project in our laboratory to obtain resveratrol analogues with an epoxide function. To our surprise, the main product of the reaction was spectroscopically characterized as 2-hydroxy-3,5,4'-trimethoxystilbene (**5**), indicating a direct aromatic hydroxylation at ring A of the stilbene nucleus, without epoxidation of the central double bond (Scheme 1). Hydroxylation at positions 2 and 4 suggested us that this unexpected reaction could be an aromatic electrophilic substitution, where the activated positions, namely those in *ortho* and *para* to the methoxy groups, regioselectively oriented the formation of the products.

This was easily confirmed by a calculation of electrostatical charges obtained through semi-empiric minimization with force field PM3 (Figure 2). In fact, in the model compound **2**, the electronic charge density is higher at C-2, C-4 and C-6 (3,5-dimethoxy substituted ring) whereas is lower at positions C-2', C-3', C-5', C-6' (4'-methoxy substituted ring).

Scheme 1.



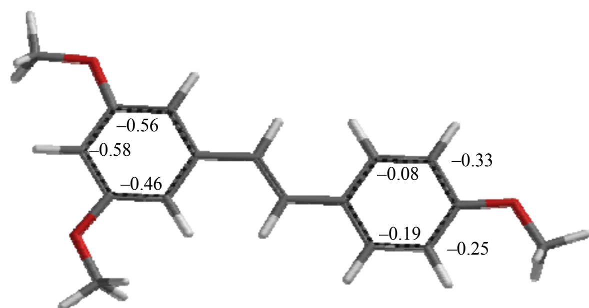


Figure 2: electronic charge density for **2**

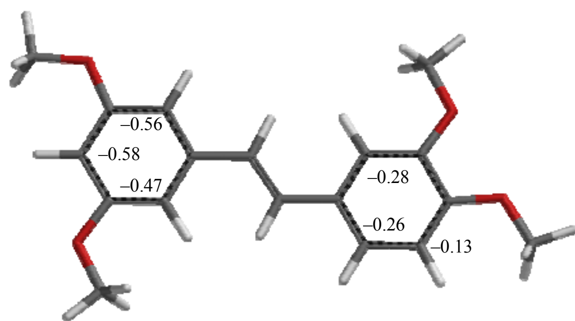


Figure 3: electronic charge density for **10**

In addition, a recent article about the synthesis of methoxy-substituted phenols by peracid oxidation presented convincing evidences for a two-steps electrophylic substitution mechanism [30].

To test the applicability of this fast, smooth hydroxylation method of methoxystilbenes we synthetised further two substrates bearing 3,5-dimethoxy groups on rings A and 3,5 or 3,4-dimethoxy groups on ring B.
According to the above cited methodology (Wittig olefination) the reaction of 3,5-dimethoxy-benzaldehyde or 3,4-dimethoxybenzaldehyde and diethyl (3,5-dimethoxybenzyl) phosphonate was carried out to obtain respectively the 3,5,3',5'-tetramethoxystilbene (**7**) and 3,5,3',4'-tetramethoxystilbene (**10**).

Analogoulsy to **2**, the substrate **7** also reacted very quickly, at rt, with *m*-CPBA to give a reaction mixture which remained unmodified after prolonged time, with a 68% conversion. Two main products were isolated and spectroscopically characterized as 2-hydroxy-3,5,3',5'-tetramethoxystilbene (**8**) and 4-hydroxy-3,5,3',5'-tetramethoxystilbene (**9**) (see Scheme 1). Finally, when **10** was treated with m-CPBA under the same conditions employed for **7**, a reaction mixture was rapidly obtained, which remained unmodified after prolonged time, with a 66 % conversion. The main products, after spectral characterization, were established as 2-hydroxy-3,5,3',4'-tetramethoxystilbene (**11**) and 4-hydroxy-3,5,3',5'-tetramethoxystilbene (**12**) (see Scheme 1). This in agreement with the electronic charge density calculations, showing higher values at C-2, C-4 and C-6 and lower values at C-2', C-5' and C-6' (Figure 3).

Compounds **5**, **8** and **11** are new. Compound **9** has been previously obtained by Wittig olefination at –78 °C under nitrogen, using 3,5-dimethoxybenzyltriphenylphosphonium bromide and a protected aldehyde; final deprotection gave a mixture of the *cis/trans*-stilbenes (3:7) [28]; also compound **12** has been previously obtained by Knoevenagel condensation [29]. It is also worthy of note here that compound **6** has been recently reported as a putative metabolite of 3,4,5,4'-tetramethoxystilbene (DMU-212), this latter being under preclinical evaluation as a potential anti-tumor prodrug that undergoes metabolic activation by cytochrome P450 enzymes affording hydroxylated metabolic products [13, 31]. This suggests that hydroxylated polymethoxystilbenes are good candidates as potential antitumor agents.

On the basis of these results, and the present interest towards antiangiogenic agents, we carried out evaluation of compounds **5**, **8** and **11** employing the angiogenesis bioassay previously developed by some of us [20-23], as a part of our studies on the antitumor/antiangiogenic properties of methylated resveratrol analogues. These previously unreported compounds were selected on the basis of their higher stability and larger availability with respect to their 4-hydroxy analogues (**6**, **9** and **12**). The trimethoxystilbene **2** was also included as a reference compound.

The potential of compounds **5**, **8** and **11** to interfere with vessel growth has been investigated by means of an angiogenesis bioassay set up in our lab [23]. This method consists of a three dimensional fibrin gel support in which we include dextran microcarriers beads with adhering porcine aortic endothelial cells (AOC). The AOC growth was assessed after 48 and 96 h of incubation. Our data evidenced that all the compounds under text, except **11**, exerted an inhibitory effect ($p<0.001$) on the angiogenic process at all the concentration tested. With regard to

compound **11**, our data evidenced that the lowest concentration, which is ineffective after 48 h, became effective (p<0.001) after 96 h even if showing a weaker effect (p<0.001) in comparison to the other compounds. Moreover, 1 and 10 μM of compound **11**

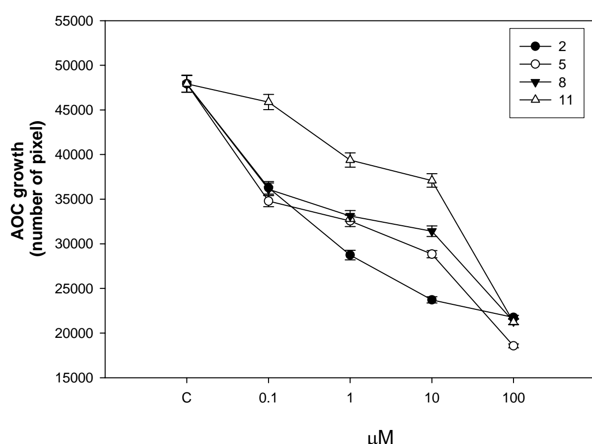


Figure 4: Effect of the treatment with compounds **2**, **5**, **8** and **11** (0.1, 1, 10 and 100 μM) for 48 h on AOC growth. Data represent mean ± SEM of 4 replicates/treatment repeated in 4 different experiments

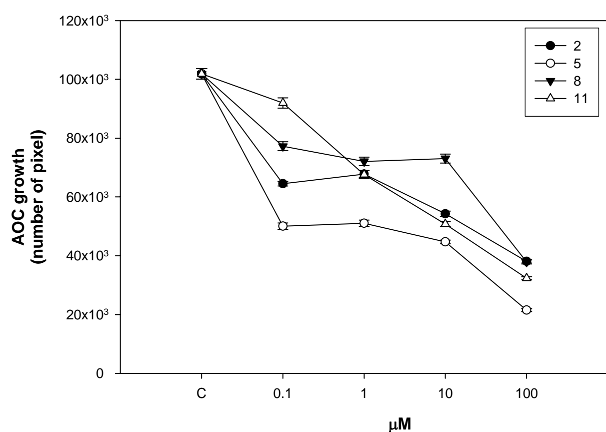


Figure 5: Effect of the treatment with compounds **2**, **5**, **8** and **11** (0.1, 1, 10 and 100 μM) for 96 h on AOC growth. Data represent mean ± SEM of 4 replicates/treatment repeated in 4 different experiments.

exerted a significant inhibitory effect (p<0.001) after 48 h of incubation although displaying a lesser potency; after the 96 h incubation time, the effectiveness of **11** resulted enhanced, thus suggesting that this compound could require a longer time to exert its effect. Overall, after the 48 h incubation, compounds **2,** at the concentration 1 and 10 μM, and **5**, at 100 μM, resulted the most potent (p<0.001). More interestingly, after 96 h, compound **5** appeared the most effective (p<0.001), since it displayed the most relevant suppressive effect at the concentrations of 1, 10 and 100 μM (Figure 4 and 5; Photos 1-4).

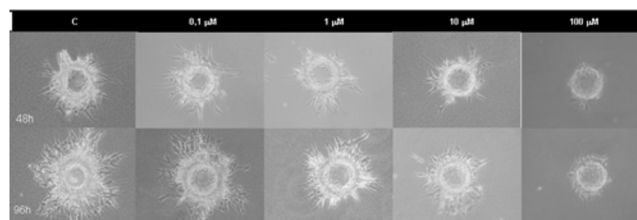


Photo 1: Phase contrast micrographs showing AOC growth at 48 h and 96 h in fibrin gel matrix. Cells were cultured in CM or treated with compound **2** at the concentrations of 0.1, 1, 10 and 100 μM.

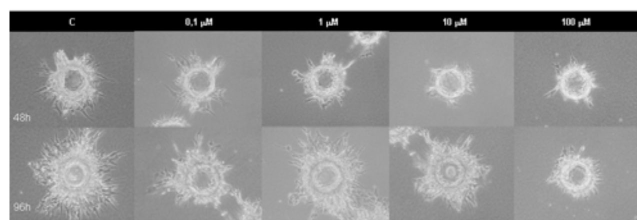


Photo 2: Phase contrast micrographs showing AOC growth at 48 h and 96 h in fibrin gel matrix. Cells were cultured in CM or treated with compound **5** at the concentrations of 0.1, 1, 10 and 100 μM.

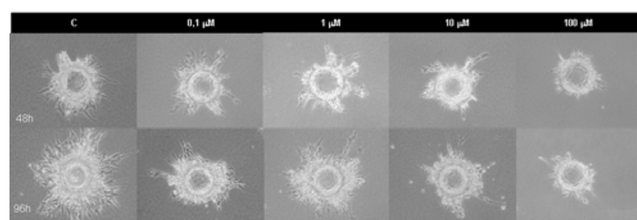


Photo 3: Phase contrast micrographs showing AOC growth at 48 h and 96 h in fibrin gel matrix. Cells were cultured in CM or treated with compound **8** at the concentrations of 0.1, 1, 10 and 100 μM.

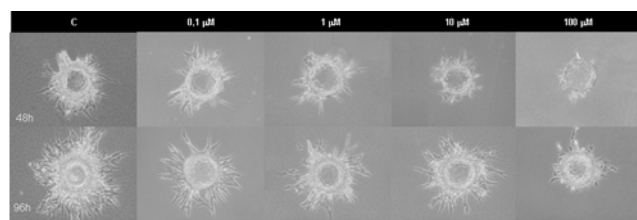


Photo 4: Phase contrast micrographs showing AOC growth at 48 h and 96 h in fibrin gel matrix. Cells were cultured in CM or treated with compound **11** at the concentrations of 0.1, 1, 10 and 100 μM

To the best of our knowledge, this is the first application of a direct aromatic hydroxylation by *m*-CPBA to polymethoxystilbenes. The method here reported, although limited in % conversion and product yield, could be of valuable help for the rapid and mild preparation of limitate amounts of these hydroxylated derivatives, allowing a first-step biological evaluation. In addition, this methodology is of potential usefulness in direct ring hydroxylation of many substrates bearing an aromatic ring with positions highly activated to the aromatic electrophilic substitution. The easy availability of compounds **5**, **8** and **11** allowed us to test these

compounds as potential antiangiogenic agents, in comparison with **2**.

In our assay, the 3,5,4'-trimethoxystilbene **2** was confirmed as a potent antiangiogenic agent determining till to a 65% reduction in new vessel growth. More interesting, its 2-hydroxy analogue **5** resulted even more active since after 96 h of incubation, at the highest concentration tested, it produced a 80% inhibition in neovascularization. Moreover, the other stilbenoids (**8** and **11**) tested in the angiogenesis bioassay have been demonstrated to be able to negatively control new vessel growth even if to a lesser extent then compounds **2** and **5**. In particular, compound **11** showed a marked increase of the activity from 48h to 96 h incubation.

These data suggest that the resveratrol-like substitution (3,5,4'-trimethoxy) is the more effective in inhibition of new vessel growth, and the presence of a further hydroxy group may have a role in improving the effectiveness with time, for instance because of an higher diffusion in a hydrophilic medium. In the presence of four methoxy groups and one hydroxy group, as in compounds **8** and **11**, the 3,5-dimethoxy substitution pattern appears more effective than the 3,4-dimethoxy pattern; of course, further data are needed for a deeper understanding of the structure-activity relationships for these resveratrol analogues. In conclusion, these data, although preliminary, may be of interest in view of possible optimization of these stilbenoids as antiangiogenic agents for therapeutic use.

Experimental

General method: All reagents were of commercial quality and were used as received (Merck and Sigma-Aldrich); all reagents for angiogenesis bioassay were obtained from Sigma unless otherwise specified. Solvents were distilled using standard techniques. *m*-Chloroperbenzoic acid (*m*-CPBA) of 99% assay was obtained by washing the commercial 77% material (Aldrich) whit a phosphate buffer of pH 7.5, and drying the residue under reduced pressure. Melting points were determined on a Kofler apparatus. Mass spectra were recorded in ESI positive mode on a Micromass ZQ2000 spectrometer (Waters). ^{1}H and ^{13}C NMR spectra were run on a Varian Unity Inova spectrometer at 500 and 125 MHz, respectively, in $CDCl_3$ or C_6D_6 solutions with TMS as internal standard. *J* values are given in Hertz. IR spectra were taken with an Perkin-Elmer Spectrum BX FT-IR System spectrophotometer using CCl_4 as solvent. UV spectra were recorded on a double-ray Lambda 25 spectrophotometer (Perkin-Elmer) using CH_3CN as solvent. All reactions were monitored by TLC on commercially available precoated plates (silica gel 60 F 254) and the products were visualized with cerium sulphate solution. Silica gel 60 was employed for column chromatography. Elemental analyses were performed on a Perkin-Elmer 240B microanalyzer.
Compounds **2**, **7** and **10** were synthesized through an Arbuzov rearrangement followed by a Horner-Emmons-Wadsworth reaction, as previously reported [27]. According to a previously reported method, this procedure affords the *E*-stilbenoid with a minimal percentage of its *Z*-isomer [32].

Synthesis of 3,5,4'-tri-methoxystilbene (2): 4-methoxybenzylchloride (1.1 mL, 8.13 mmol) was heated with excess of triethyl phosphite (1.85 mL, 10.6 mmol) to 130 °C to give diethyl (4-methoxybenzyl)phosphonate. This latter was cooled to 0 °C, and dry DMF (10 mL) and 0.41g (7.6 mmol) of sodium methoxide were added. To this solution, 1.18 g (7.1 mmol) of 3,5-dimethoxybenzaldehyde was added and the mixture was allowed to stand at room temperature for 1 h. Then it was heated to 100 °C and allowed to stand at this temperature for 1 h. After cooling, the reaction mixture was stirred overnight and subsequently quenched with water and extracted with diethyl ether. The combined organic layers were washed with water and dried over Na_2SO_4, affording 1.57 g of the product **2** (82.3 % yield); white solid mp 55 - 56 °C (MeOH); ESIMS: 271 $[M+H]^+$; ^{1}H and ^{13}C NMR data are in agreement with literature data [33].

Synthesis of 3,5,3',5'-tetramethoxystilbene (7): 3,5-dimethoxybenzylbromide (1.72 g, 11 mmol) was heated with excess of triethyl phosphite (4 mL, 10.8 mmol) to 130 °C to give diethyl (4-methoxybenzyl)phosphonate. This latter was cooled to 0° C, and dry DMF (20 mL) followed by 0.81 g (15 mmol) of sodium methoxide were added. To this solution, 1.92 g (11.5 mmol) of 3,5-dimethoxybenzaldehyde was added and the mixture was allowed to stand at room temperature for 1 h. Then it was heated to 100 °C and allowed to stand at this temperature for 1 h. After cooling, the reaction mixture was stirred overnight and subsequently quenched with water-methanol (2:1) affording 2.93 g of the product **7** (88.8 % yield) as white solid, mp 131-132 °C (MeOH); ESIMS: 301 $[M+H]^+$; NMR and IR data are in agreement with literature data [34, 35].

Synthesis of 3,5,3',4'-tetramethoxystilbene (10): 3,5-dimethoxybenzylbromide (1.70 g, 7.3 mmol) was heated with excess of triethyl phosphite (1.26 mL, 7.3 mmol) to 130 °C to give diethyl (4-methoxybenzyl)phosphonate. This latter was cooled to 0° C, and dry DMF (10 mL) followed by 0.432 g (8 mmol) of sodium methoxide were added. To this solution, 1.39 g (8.3 mmol) of 3,4-dimethoxybenzaldehyde was added and the mixture was allowed to stand at room temperature for 1 h. Then it was heated to 100 °C and allowed to stand at this temperature for 1 h. After cooling, the reaction mixture was stirred overnight and subsequently quenched with water and extracted with CH_2Cl_2. The combined organic layers were washed with water and dried over Na_2SO_4, affording 1.92 g of the product **10** (87.6 % yield) as white solid, mp 63 – 64 °C (MeOH); ESIMS: *m/z* 301 $[M+H]^+$; NMR data are in agreement with literature data [36].

General Procedure for hydroxylation of compounds 2, 7 and 10 with *m*-CPBA: To a stirred solution of the substrate in CH_2Cl_2 (0.105 mmol/mL) a solution of *m*-CPBA in CH_2Cl_2 (0.150 mmol/mL) was added at room temperature. Then, the reaction mixtures were washed with a $NaHSO_3$ solution and subsequently with saturated aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4), filtered, and concentrated *in vacuo*; the residues were submitted to flash-chromatography on 3 x 25 cm silica gel column, eluted with EtOAc in *n*-hexane (from 0 % to 30 %).

2-Hydroxy-3,5,4'-trimethoxystilbene (5)

White solid; mp 100-102 °C (MeOH), 21% yield.
R_f (*n*-hexane/EtOAc 70:30) 0.58.
IRν_{max}: 3558, 3003, 2954, 2938, 2868, 1605, 1511, 1489, 1465, 1431, 1281, 1252, 1237, 1200, 1174, 1151, 1082, 1057, 1041, 966, 926, 826 cm^{-1}.
UV λ_{max} (ε): 217 (3.04 E+04), 303 (2.99 E+04).
^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.82 (s, 3H, 4'-OCH_3), 3.83 (s, 3H, 5-OCH_3), 3.88 (s, 3H, 3-OCH_3), 5.54 (bs, 1H, OH), 6.40 (d, *J* 2.5, 1H, H-4), 6.66 (d, *J* 2.5, 1H, H-6), 6.90 (d, *J* 8.5, 2H, H-2' and H-6'), 7.07 (d, *J* 16.5, 1H, H-α), 7.28 (d, *J* 16.5, 1H, H-β), 7.47 (d, *J* 8.5, 2H, H-3' and H-5').
^{13}C NMR ($CDCl_3$, 125 MHz) δ 55.1 (3-OCH_3), 55.6 (4'-OCH_3), 55.9 (5-OCH_3), 98.3 (C-4), 100.7 (C-6), 113.9 (C-3' and C-5'), 120.8 (C-α),123.5 (C-1'), 127.6 (C-2' and C-6'), 128.7 (C-β), 130.5 (C-1), 137.6 (C-5), 147.2 (C-3), 152.9 (C-2), 159.1 (C-4'). ESIMS: 309 $[M+Na]^+$, 595 $[2M+Na]^+$. Anal. Calcd for $C_{17}H_{18}O_4$: C, 71.31; H, 6.34. Found: C, 71.45; H, 6.32.

4-Hydroxy-3,5,4'-tri-methoxystilbene (6)

Pale-yellow amorphous powder; 8.5% yield.
R_f (*n*-Hexane/EtOAc 70:30) 0.32.
IR ν_{max}: 3557, 2955, 2940, 2838, 1601, 1594, 1490, 1465, 1430, 1237, 1205, 1153, 1057, 965, 929, 828 cm^{-1}.
UV λ_{max} (ε): 303 (2.03 E+04).
^{1}H NMR (C_6D_6, 500 MHz) δ 3.31 (s, 3H, 4'-OCH_3), 3.37 (s, 6H, 3-OCH_3 and 5-OCH_3), 5.37 (bs, 1H, OH), 6.66 (s, 2H, H-2 and H-6), 6.83 (d, *J* 8.0, 2H, H-2' and H-6'), 6.95 (d, *J* 16.0, 1H, H-α), 7.01 (d, *J* 16.0, 1H, H-β), 7.39 (d, *J* 8.0, 2H, H-3' and H-5').
^{13}C NMR ($CDCl_3$, 125 MHz) δ 55.4 (4'-OCH_3), 56.3 (3-OCH_3 and 5-OCH_3), 103.1 (C-2 and C-6), 114.2 (C-3' and C-5'), 126.2 and 126.7 (C-α and C-β), 127.7 (C-2' and C-6'), 130.2 (C-1), 131.1 (C-1'), 134.5 (C-4), 147.4 (C-3 and C-5), 159.1 (C-4').

2-Hydroxy- 3,5,3',5'-tetramethoxystilbene (8)

White solid; mp 119-121°C (MeOH); 23% yield.
R_f (*n*-Hexane/EtOAc 70:30) 0.46.
IR ν_{max}: 3557, 2955, 2940, 2838, 1601, 1594, 1490, 1465, 1430, 1237, 1205, 1153, 1057, 965, 929, 828 cm^{-1}.
UV λ_{max} (ε): 2.27 (2.30 E+04), 303 (2.03 E+04).
^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.82 (s, 3H, 5-OCH_3), 3.83 (s, 6H, 3'-OCH_3 and 5'-OCH_3), 3.88 (s, 3H, 3-OCH_3), 5.57 (bs, 1H, OH), 6.39 (d, *J* 2.5, 1H, H-4), 6.66 (d, *J* 2.5, 1H, H-6), 6.70 (d, *J* 2.0, 2H, H-2' and H-6'), 7.09 (d, *J* 16.5, 1H, H-α), 7.39 (d, *J* 16.5, 1H, H-β).
^{13}C NMR ($CDCl_3$, 125 MHz) δ 55.4 (3'-OCH_3 and 5'-OCH_3), 55.7 (5-OCH_3), 56.1 (3-OCH_3), 98.9 (C-4'), 100.0 (C-4), 101.0 (C-6), 104.6 (C-2' and C-6'), 123.0 (C-α), 123.5 (C-1'), 129.3 (C-β), 138.08 (C-1), 139.8 (C-5), 147.3 (C-3), 153.2 (C-2), 160.9 (C-3'). ESIMS: 317 $[M+H]^+$, 339 $[M+Na]^+$; Anal. Calcd for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37. Found: C, 68.27; H, 6.49.

4-Hydroxy- 3,5,3',5'-tetramethoxystilbene (9)

White amorphous powder; 10.5 % yield.
R_f (*n*-hexane/EtOAc 70:30) 0.25.
ESIMS: 317 $[M+H]^+$, 355 $[M+K]^+$.
NMR and IR data are in agreement with literature data [28].

2-Hydroxy- 3,5,3',4'-tetramethoxystilbene (11)

White solid; mp 124-125 °C (MeOH); 19 % yield.
R_f (*n*-Hexane/EtOAc 70:30) 0.35.
IR ν_{max}: 3554, 2998, 2940, 2833, 1601, 1544, 1492, 1246, 1205, 1154, 1058, 976, 929, 830 cm^{-1}.
UV λ_{max} (ε): 220 (2.40 E+04), 326 (2.2 E+04).
^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.82 (s, 3H, 5-OCH_3), 3.88 (s, 3H, 3-OCH_3), 3.90 (s, 3H, 4'-OCH_3), 3.94 (s, 3H, 3'-OCH_3), 5.56 (bs, 1H, OH), 6.42 (d, *J* 2.5, 1H, H-4), 6.67 (d, *J* 2.5, 1H, H-6), 6.86 (d, *J* 8.0, 1H, H-5'), 7.08 (d, *J* 8.0, 1H, H-6'), 7.10 (bs, 1H, H-2'), 7.35 (d, *J* 16.0, 1H, H-β), 7.81 (d, *J* 16.0, 1H, H-α); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 55.7 (5-OCH_3), 55.8 (4'-OCH_3), 55.9 (3'-OCH_3), 56.0 (3-OCH_3), 98.5 (C-4), 100.8 (C-6), 108.8 (C-2'), 111.2 (C-5'), 119.9 (C-6'), 121.6 (C-α), 123.4 (C-1), 129.1 (C-β), 130.9 (C-1'), 137.7 (C-2), 147.3 (C-3), 148.8 (C-4'); 149.0 (C-3'); 153.0 (C-5).
ESIMS: *m/z* 317 $[M+H]^+$, 339 $[M+Na]^+$; Anal. Calcd for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37. Found: C, 68.31; H, 6.52.

4-Hydroxy- 3,5,3',4'-tetramethoxystilbene (12)

White amorphous powder; 5.2 % yield.
R_f (*n*-hexane/EtOAc 70:30) 0.25;
ESIMS: 317 $[M+H]^+$, 355 $[M+K]^+$.
NMR and IR data are in agreement with literature data [29].

Endothelial cell culture: An immortalized porcine aortic endothelial cell line (AOC) [37] was generously provided by José Yelamos (Department of Immunology, IMIM-Hospital del Mar, Barcelona Biomedical Research Park, Barcelona, Spain). In all experiments, AOC at 19th passage were used and seeded in culture medium (CM) composed by M199 supplemented with sodium bicarbonate (2.2 mg/ml), bovine serum albumin (BSA 0.1%), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), selenium (5 ng/ml) and transferrin (5 μg/ml).

Three-dimensional endothelial cell culture on a fibrin gel support: The microcarrier-based fibrin gel angiogenesis assay was performed as described by Basini *et al*. [23] Briefly, 12.5 mg gelatin-coated cytodex-3 microcarriers in 1 ml PBS were incubated for 3 h to hydrate. After two washings in PBS and one in CM, the microcarriers were put in flasks containg 5 ml CM; AOC (5 x 10^5) were added and cultured for 24 h in order to let the endothelial cells coat the microcarriers. For the fibrin gel preparation, 40 μl microcarriers covered by AOC were pipetted into 6 well plates containing a solution of fibrinogen (1mg/ml PBS, pH 7.6), added with 1250 IU thrombine (250 μl). Fibrin gels were allowed to polymerize for 30 min at 37 °C, then they were equilibrated for 60 min with 2 ml M199. After a change of the medium, AOC were treated with VEGF (100 ng/ml; PeproTech EC Ltd, London, UK) in the presence or absence of 0.1 1, 10 or 100 μM of **2**, **5**, **8** or **11**. Plates were incubated at 37°C under humidified atmosphere (5% CO_2). AOC were cultured for 96 h, renewing totally the treatment after 48h.

Quantification of AOC growth on fibrin gel matrix: Endothelial cell proliferation in the fibrin gel matrix was evaluated by means of the public domain NIH Program Scion Image Beta 4.02 (Scion Corporation, MA, USA, http://rsb.info.nih.gov/nih-image/). Ten pictures were taken for each gel at 48 and 96 h; images were converted into gray scale, resized to 50% (Paintbrush Software, MS Office) and saved as Bitmap 24bit format compatible with Scion. The modified images were then imported into the program and measurements were made drawing the perimeter of the area occupied by AOC expressed as number of pixel. In order to validate the measurement of the area covered by AOC in fibrin gels as a reliable method to evaluate cell proliferation, fibrin gels were stained by the nuclear dye bis-benzimide (Hoechst 33258, 20 μg/ml in PBS for 60 min) and examined by the fluorescence microscope [21, 22]. This procedure was performed 20 times; for each experiment the number of nuclei was counted under fluorescence and pictures of the aera covered by AOC were taken in order to measure the surface covered in the fibrin gel. A strong correlation was observed between the area covered by AOC and the number of nuclei found in the same area ($r = 0.96$).

Statistical analysis: Bioassays were repeated at least 4 times (4 replicates/treatment). Experimental data are presented as mean ± SEM; statistical differences between treatments were calculated with Multifactorial ANOVA using Statgraphics package (STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by Scheffè's F test; p values < 0.05 were considered to be statistically significant.

Acknowledgements – Financial support for this work was obtained from the University of Catania (PRA, Catania, Italy) and from MIUR (PRIN, Rome,

Italy). We are grateful to Professors Vincenzo Amico and Antonio Rescifina (University of Catania, Italy) as well as to Prof. Placido Neri (University of Salerno, Italy) for helpful discussions about this manuscript. We would like to thank Professor Yelamos (Department of Immunology, IMIM-Hospital del Mar, Barcelona Biomedical Research Park, Barcelona, Spain) for supplying AOC.

References

[1] Takaoka MJ. (**1940**) Phenolic substances of white hellebore (*Veratrum grandiflorum* Loes. fil.). *J.-FE. Sci. Hokkaido Imp. Univ., **3***, 1-16.

[2] Nonomura S, Kanagawa H, Makimoto A. (**1963**) Chemical constituents of polygonaceous plants. I. Components of *Polygonum cuspidatum. Yakugaku Zasshi, **83***, 988-990.

[3] Renaud S, de Lorgeril M. (**1992**) Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet, **339***, 1523-1526.

[4] Bradamante S, Barenghi L, Villa A. (**2004**) Cardiovascular protective effects of resveratrol. *Cardiovascular Drug Reviews, **22***, 169-188.

[5] Aggarwal BB, Shishodia S. (**2006**) Oxidative Stress and Disease Vol ***20***. *Resveratrol in Health and Disease*. Taylor & Francis, London. 1–712 pp.

[6] Jeandet P, Bessis R, Sbaghi M, Meunier P, Trollat P. (**1995**) Resveratrol content of wines of different ages: relationship with fungal disease pressure in the vineyard. *American Journal of Enology and Viticulture*, ***46***, 1-4.

[7] Baur JA, Sinclair DA. (**2006**) Therapeutic potential of resveratrol: the in vivo evidence. *Nature Reviews Drug Discovery*, ***5***, 493-506.

[8] Burjonroppa S, Fujise K. (**2006**) Resveratrol as cardioprotective agent: evidence from bench and bedside. In *Resveratrol in Health and Disease*, Aggarwal BB, Shishodia S. (Eds). Taylor & Francis, London, 539–555.

[9] Signorelli P, Ghidoni R. (**2005**) Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. *Journal of Nutritional Biochemistry*, ***16***, 449-466.

[10] Delmas D, Lançon A, Colin D, Jannin B, Latruffe N. (**2006**) Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. *Current Drug Targets*, ***7***, 423-442.

[11] Jazirehi AR, Bonavida B. (**2006**) Resveratrol as a sensitizer to apoptosis-inducing stimuli. In *Resveratrol in health and disease.* Aggarwal BB, Shishodia S. (Eds). Taylor & Francis, London, 399-421.

[12] Chillemi R, Sciuto S, Spatafora C, Tringali C. (**2007**) Anti-tumor properties of stilbene-based resveratrol analogues: Recent results. *Natural Product Communications*, ***2***, 499-513.

[13] Sale S, Verschoyle RD, Boocock D, Jones DJL, Wilsher N, Ruparelia KC, Potter GA, Farmer PB, Steward WP, Gescher AJ. (**2004**) Pharmacokinetics in mice and growth-inhibitory properties of the putative cancer chemopreventive agent resveratrol and the synthetic analogue *trans* 3,4,5,4'-tetramethoxystilbene. *British Journal of Cancer*, **90**, 736-744.

[14] Tron GC, Pirali T, Sorba G, Pagliai F, Busacca S, Genazzani AA. (**2006**) Medicinal chemistry of combretastatin A4: Present and future directions. *Journal of Medicinal Chemistry*, ***49***, 3033-3044.

[15] Belleri M, Ribatti D, Vicoli S, Cotelli F, Forti L, Tannini V, Stivala LA, Presta M. (**2005**) Antiangiogenic and vascular-targeting activity of the microtubule-destabilizing trans-resveratrol derivative 3,5,4'-trimethoxystilbene. *Molecular Pharmacology*, ***67***, 1451-1459.

[16] Grasselli F, Basini G, Bussolati S, Tamanini C. (**2002**) Effect of reduced oxygen tension on reactive oxygen species production and activity of antioxidant enzymes in swine granulosa cells. *Reproduction in Domestic Animals*, ***37***, 362-368.

[17] Grasselli F, Basini G, Tirelli M, Cavalli V, Bussolati S, Tamanini C. (**2003**) Angiogenic activity of porcine granulosa cells co-cultured with endothelial cells in a microcarrier-based three-dimensional fibrin gel**.** *Journal of Physiology and Pharmacology*, ***54***, 361-370.

[18] Grasselli F, Basini G, Bianco F, Tirelli M, Tamanini C. (**2004**) Effect of reduced oxygen tension on reactive oxygen species production and activity of antioxidant enzymes in swine granulosa cells. *Biofactors*, ***20**,* 61-69.

[19] Basini G, Bianco F, Grasselli F, Tirelli M, Bussolati S, Tamanini C. (**2004**) The effects of reduced oxygen tension on swine granulosa cell. *Regulatory Peptides*, ***120***, 69-75.

[20] Bianco F, Basini G, Grasselli F. (**2005**) Angiogenic activity of swine granulosa cells: effects of hypoxia and vascular endothelial growth factor Trap R1R2, a VEGF blocker *Domestic Animal Endocrinology*, ***28**,* 308-319.

[21] Basini G, Bussolati S, Santini SE, Grasselli F. (**2007**) Sanguinarine inhibits VEGF-induced angiogenesis in a fibrin gel matrix. *Biofactors*, ***29***, 11-18.

[22] Basini G, Bussolati S, Santini SE, Bianchi F, Careri M, Mangia A, Musci M, Grasselli F. (**2007**) Anti-angiogenesis in swine ovarian follicle: A potential role for 2-methoxyestradiol. *Steroids*, ***72***, 660-665.

[23] Basini G, Bussolati S, Santini SE, Bianchi F, Careri M, Mangia A, Musci M, Grasselli F. (**2008**) Hydroxyestrogens inhibit angiogenesis in swine ovarian follicles. *Journal of Endocrinology*, ***199**,* 127-135.

[24] Kerbel R, Folkman J. (**2002**) Clinical translation of angiogenesis inhibitors. *Nature Reviews Cancer*, ***2***, 727-739.

[25] Fernando NH, Hurwitz HI. (**2003**) Inhibition of vascular endothelial growth factor in the treatment of colorectal cancer. *Seminars in Oncology*, ***30***, 39-50.

[26] Cardile V, Lombardo L, Spatafora C, Tringali C. (**2005**) Chemo-enzymatic synthesis and cell-growth inhibition activity of resveratrol analogues. *Bioorganic Chemistry,* ***33***, 22-33.

[27] Cardile V, Chillemi R, Lombardo L, Sciuto S, Spatafora C, Tringali C. (**2007**) Antiproliferative activity of methylated analogues of *E*- and *Z*-resveratrol. *Zeitschrift fur Naturforschung C,* ***62***, 189-195.

[28] Roberti M, Pizzirani D, Recanatini M, Simoni D, Grimaudo S, Di Cristina A, Abbadessa V, Gebbia N, Tolomeo M. (**2006**) Identification of a Terphenyl Derivative that Blocks the Cell Cycle in the G0-G1 Phase and Induces Differentiation in Leukemia Cells. *Journal of Medicinal Chemistry*, ***49***, 3012-3018.

[29] Zhang L, Gellersted G. (**1994**) Reactive structures in wood and high-yield pulps. IV. Daylight-induced oxidation of stilbene structures in the solid state. *Acta Chemica Scandinavica*, ***48***, 490-497.

[30] Bjorsvik H-R, Occhipinti G, Gambarotti C, Cerasino L, Jensen VR. (**2005**) Synthesis of methoxy-substituted phenols by peracid oxidation of the aromatic ring. *Journal of Organic Chemistry*, ***70***, 7290-7296.

[31] Potter GA, Butler PC, Ruparelia KC, Ijaz T, Wilsher NC, Wanogho E, Tan HL, Hoang TTV, Stanley LA, Burke MD. (**2002**) The cancer preventative agent resveratrol is converted to the anticancer agent piceatannol by the cytochrome P450 enzyme CYP1B1. *British Journal of Cancer,* ***86S***, 117.

[32] Bachelor FW, Loman AA, Snowdon LR. (**1970**) Synthesis of pinosylvin and related heartwood stilbenes. *Canadian Journal of Chemistry*, ***48***, 1554-1557.

[33] Mannila E, Talvitie A, Kolehmainen E. (**1993**) Antileukemic compounds derived from stilbenes in *Picea abies* bark. *Phytochemistry*, ***33*****,** 813-816.

[34] Murias M, Handler N, Erker T, Pleban K, Ecker G, Saiko P, Szekeres T, Jäger W. (**2004**) Resveratrol analogues as selective cyclooxygenase-2 inhibitors: synthesis and structure-activity relationship. *Bioorganic Medicinal Chemistry*, ***12***, 5571-5578.

[35] Li Y-Q, Li Z-L, Zhao W-J, Wen R-X, Meng Q-W, Zeng Y. (**2006**) Synthesis of stilbene derivatives with inhibition of SARS coronavirus replication. European *Journal of Medicinal Chemistry*, ***41***, 1084-1089.

[36] Kim S, Ko H, Park JE, Jung S, Lee SK, Chun Y–J. (**2002**) Design, synthesis, and discovery of novel *trans*-stilbene analogues as potent and selective human cytochrome P450 1B1 inhibitors. *Journal of Medicinal Chemistry*, ***45***, 160-164.

[37] Carrillo S, Chamorro M, Rodriguez-Gago B, Alvarez MJ, Molina JI, Rodriguez-Barbosa A, Sanchez P, Ramirez A, Munoz J, Dominguez J, Parrilla P, Yelamos J. (**2002**) Isolation and characterization of immortalized porcine aortic endothelial cell lines. *Veterinary Immunology and Immunopathology*, ***89,*** 91-98.

ALLEGATO I

“Biological effects on granulosa cells of hydroxylated and metylated resveratrol analogues”

Mol. Nutr. Food Res. 2010. 54: S236-S243.
doi:10.1002/mnfr.200900320

For legal reasons this is the LAST chance for you to correct your paper

Research Article

Biological effects on granulosa cells of hydroxylated and methylated resveratrol analogues

Giuseppina Basini[1], Corrado Tringali[2], Laura Baioni[1], Simona Bussolati[1], Carmela Spatafora[2] and Francesca Grasselli[1]

[1] Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti – Sezione di Fisiologia, Italy
[2] Dipartimento di Scienze Chimiche, Università di Catania, Catania, Italy

Several resveratrol analogues have been designed to improve bioactivity: among these polymethoxystilbenes appear to be particularly promising. The present study was set up to investigate the biological functions of polymethoxystilbenes **2** and **3**, recently found in our labx as antiangiogenic agents, on a well-defined swine granulosa cell model. Proliferative activity and effects on steroidogenesis were evaluated, as well as the effect on granulosa cell VEGF production, since these cells in basic conditions synthesize the main proangiogenic peptide. Moreover, we considered the effect of these two resveratrol analogues on granulosa cell redox status. Analogue **3** inhibited granulosa cell growth, while it stimulated steroidogenesis. A similar effect was displayed by **2** on estradiol 17β production and cell proliferation at the highest concentration tested. On the other hand, at the same dosage **2** decreased progesterone levels. Both analogues inhibited VEGF output. Granulosa cell redox status was unaffected by resveratrol analogue **2** while the highest concentration of **3** stimulated free radicals generation and scavenging enzyme activities. The overall results indicate that analogue **3** is the more powerful compound, thus suggesting that a slight modification in the structure markedly increases effectiveness. These data could be useful to develop more active resveratrol analogues for therapeutic use.

Received: July 7, 2009
Revised: August 6, 2009
Accepted: August 24, 2009

Keywords:
Free radicals / Polymethoxystilbenes / Resveratrol analogues / Steroids / VEGF

UNCORRECTED PROOF

1 Introduction

Apples, onions, chocolate, green tea and other plant extracts are good sources of phenolics. Resveratrol (3,5,4′-trihydroxystilbene **1**; Fig. 1) is a naturally occurring stilbene derivative, found in mulberries, peanuts, in some medicinal plants and mainly present in skin of grapes and thus in red wine [1]. Resveratrol is reported to be a natural chemopreventive agent against cancer, a potent antioxidant and an anti-inflammatory molecule [2]. However, the concentrations required to exert these effects may be difficult to achieve by drinking only one or two glasses of red wine a day. Therefore, developing more potent analogues of resveratrol may provide a feasible means of achieving effective concentrations. The available *in vivo* studies indicate that resveratrol, although absorbed in high extent by the organism, has a poor bioavailability and may be converted *in vivo* into compounds that sometimes, as for piceatannol, are effective [3] but usually lacks of its activities [4]. Thus, many resveratrol analogues have been recently synthesized in the hope to increase the activity and/or the bioavailability [5]. Among these, polymethoxystilbenes are particularly interesting since the presence of a 3,5-dimethoxy moiety appears to be associated with noticeable biological activity. Polymethoxystilbenes appear as a sub-group of great interest among the resveratrol analogues [6] and a deeper evaluation of these compounds may supply new lead compounds; *in vivo* studies indicate that three- or tetramethoxystilbenes are characterized by a higher bioavailability than resveratrol

Correspondence: Professor Giuseppina Basini, Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti – Sezione di Fisiologia Veterinaria, Via del Taglio 10, 43126 Parma, Italy
Fax: +39-0521-032770
E-mail: basini@unipr.it

Abbreviations: CM, culture medium; **E2,** estradiol 17β; **P4,** progesterone; **SOD,** superoxide dismutase

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Colour in Web, Color in Print

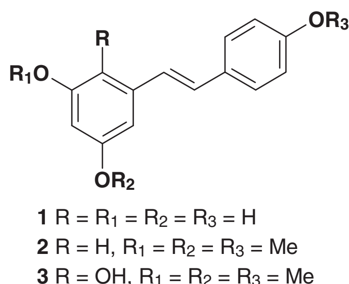


Figure 1. Structures of compounds 1–3.

[7, 8]. In a recent paper, we have documented that the resveratrol analogues 3,5,4′-trimethoxystilbene (**2**) and 2-hydroxy-3,5,4′-trimethoxystilbene (**3**) possess a powerful antiangiogenic activity [9] as evaluated by an angiogenesis bioassay set up in our lab (Fig. 1) [10]. The present research was undertaken to get an insight into the biological functions of compounds **2** and **3**. In order to study the effects of both 3,5,4′-trimethoxystilbene and its hydroxylated analogue on parameters related to cell growth, hormone production, angiogenesis and redox status swine granulosa cells were used as a previously well-defined experimental model [11–15]. Granulosa cells were chosen since they are true endocrine cells and are involved in several physiological processes such as angiogenesis. Moreover they are easy to recover. In particular, the main features of granulosa cell function, proliferation and steroidogenesis, were evaluated. In addition, we also tested the effect of these polymethoxystilbenes on VEGF production, since we previously demonstrated [16] that granulosa cell produce this pivotal angiogenic peptide. Finally, redox status of granulosa cells was explored after treatment with resveratrol analogues.

2 Materials and methods

2.1 Chemicals

All reagents used in this study were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

Compound **2** was synthesized through an Arbuzov rearrangement followed by a Horner–Emmons–Wadsworth reaction, as previously reported [17]. According to a previously reported method, this procedure affords the *E*-stilbenoid with a minimal percentage of its *Z*-isomer [18].

Compound **3** was synthesized through a mild hydroxylation with *m*-CPBA of **2** as previously reported [9]. Q2

2.2 Granulosa cell collection

Swine ovaries were collected at a local slaughterhouse from Large White cross-bred gilts, parity = 0. The stage of the cycle was unknown. Follicles were classified on a dimension-based fashion [14]. The ovaries were placed into cold PBS (4°C) supplemented with penicillin (500 IU/mL), streptomycin (500 μg/mL) and amphotericin B (3.75 μg/mL), maintained in a freezer bag and transported to the laboratory within 1 h. After a series of washings with PBS and ethanol (70%), granulosa cells were aseptically harvested by aspiration of large follicles (>5 mm) with a 26-gauge needle and released in medium containing heparin (50 IU/mL), centrifuged for pelleting and then treated with 0.9% prewarmed ammonium chloride at 37°C for 1 min to remove red blood cells. Cell number and viability were estimated using a haemocytometer under a phase contrast microscope after vital staining with trypan blue (0.4%) of an aliquot of the cell suspension. Cells were seeded in culture medium (CM) M199 supplemented with sodium bicarbonate (2.2 mg/mL), BSA (0.1%), penicillin (100 IU/mL), streptomycin (100 μg/mL), amphotericin B (2.5 μg/mL), selenium (5 ng/mL) and transferrin (5 μg/mL). Once seeded, cells were incubated in the presence or absence of compounds **2** or **3** (0.1, 1, 10 and 100 μM) and maintained for 48 h at 37°C under humidified atmosphere (5% CO_2). The treatment was identical for all experiments performed in this study.

2.3 Granulosa cell viability

A total of 2×10^5 cells were seeded in 96-well plates in 200 μL CM. Cell viability was assayed using a bioluminescent assay (ATP-lites; Packard Bioscience, Groningen, The Netherlands), which measured intracellular ATP levels as an indicator of cell numbers. ATP is a cell viability marker because it is present in all metabolically active cells and the concentration declines very rapidly when the cells undergo necrosis or apoptosis. The ATP lite-M assay system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. This is illustrated in the following reaction scheme: ATP + D-luciferin + O_2 → luciferase, Mg^{2+} → oxyluciferin + AMP + PPi + CO_2 + light. The emitted light is proportional to the ATP concentration. Briefly, 50 μL of mammalian cell lysis solution were added to 100 μL of cell suspension and the plate was shaken for 5 min in an orbital shaker at 700 rpm in order to lyse the cells and stabilize ATP. Then 50 μL of substrate solution were added to the wells and the microplate was shaken for 5 min in an orbital shaker at 700 rpm. The plate was placed in the dark for 10 min and the luminescence was measured in a luminometer (Victor, Packard Bioscience). The results were recorded in counts *per* second and the percentage of cell viability was calculated with reference to the negative control (cells without resveratrol analogues) for presentation purposes.

2.4 Granulosa cell steroid production

A total of 10^4 cells/well were seeded in 96-well plates in 200 μL CM supplemented with androstenedione (28 ng/

UNCORRECTED PROOF

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

MNFR 2009-00320 Journal Issue Number 1 Manuscript Number 2009-00320 Color Figure No. nil

mL). Culture media were then collected, frozen and stored at -20°C until progesterone (P4) and estradiol 17β (E2) determination by validated Radio Immuno Assays [19]. P4 assay sensitivity and ED_{50} were 0.24 and 1 nmol/L, respectively; E2 assay sensitivity and ED_{50} were 0.05 and 0.2 nmol/L. The intra- and inter-assay coefficients of variation were less than 12% for both assays.

2.5 Granulosa cell VEGF production

A total of 10^6 granulosa cells in 1 mL CM+1% fetal calf serum were seeded in 24-well plates and incubated for 48 h. VEGF in culture media was quantified by an ELISA (Quantikine, R&D Systems, Minneapolis, MI, USA). This assay, developed for human VEGF detection, has been validated for pig VEGF [20]. The assay sensitivity was 8.74 pg/mL, the inter- and intra-assay Coefficients of Variation were always less than 7%. Victor Reader set to read at a wavelength of 450 nm emission was used to quantify the reaction product.

2.6 Granulosa cell superoxide (O_2^-) production

O_2^- production was evaluated by WST-1 (4 - [3 - (4 - iodophenyl)–2 - (4 - nitrophenyl) - 2H–5 - tetrazolio] - 1,3-benzene disulfonate) test (Roche, Mannheim, Germany). The assay is based on the cleavage of the water-soluble tetrazolium salt, WST-1 to a yellow-orange, water-soluble formazan. Evidence exists that tetrazolium salts can be used as a reliable measure of intracellular O_2^- production [21, 22]. A total of 10^4 cells/200 μL CM were seeded in 9.7 Granulo6-well plates and incubated for 48 h. In total, 20 μL WST-1 were added to cells during the last 4 h of incubation and absorbance was then determined using the Victor reader at a wavelength of 450 nm against 620 nm.

2.7 Granulosa cell hydrogen peroxide (H_2O_2) production

H_2O_2 production was measured by an Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes, PoortGebouw, The Netherlands); the Amplex Red reagent reacts with H_2O_2 to produce resorufin, an oxidation product. Briefly, 2×10^5 cells/200 μL CM were seeded in 96-well plates and incubated for 48 h. After centrifugation for 10 min at $400 \times g$, the supernatants were discarded and cells were lysed adding cold Triton 1% in TRIS HCl (100 μL/well) and incubating on ice for 30 min. Undiluted cell lysates were used to perform the test and read against a standard curve of H_2O_2 ranging from 0.195 to 12.5 μM. The absorbance was determined with the Victor Reader using a 540 nm filter.

2.8 Granulosa cell scavenging enzyme activity

A total of 2×10^5 cells/200 μL CM were seeded in 96-well plates and incubated for 48 h. After centrifugation for 10 min. at $400 \times g$, the supernatants were discarded and cells were lysed adding cold Triton 1% in TRIS HCl (100 μL/10^5 cells) and incubating on ice for 30 min. Superoxide dismutase (SOD), catalase and peroxidase activities were assessed in cell lysates as described below.

SOD activity was determined by a SOD Assay Kit (Dojindo Molecular Technologies, Japan). Cell lysates were tested without dilution and a standard curve of catalase ranging from 0.156 to 20 U/mL was prepared. The colorimetric assay was performed measuring formazan produced by the reaction between tetrazolium salt (WST-1) and superoxide anion (O_2^-), produced by the reaction of an exogenous xantine oxidase. The remaining O_2^- is an indirect hint of the endogenous SOD activity. The absorbance was determined with Victor Reader reading at 450 nm against 620 nm.

Catalase activity was measured by an Amplex Red Catalase Assay Kit (Molecular Probes) based on the formation of an oxidation product (resorufin) derived from the reaction between H_2O_2 given in excess, and the Amplex Red reagent in the presence of horseradish peroxidase. Cell lysates were diluted 1:10 to perform the test and read against a standard curve of catalase ranging from 62.5 to 1000 mU/mL. The absorbance was determined with Victor Reader using a 540 nm filter.

Peroxidase activity was measured by an Amplex Red Peroxidase Assay Kit (Molecular Probes) based on the formation of an oxidation product (resorufin) derived from the reaction between H_2O_2 given in excess and the Amplex Red reagent. Cell lysates were used undiluted to perform the test and read against a standard curve of peroxidase ranging from 0.039 to 5 mU/mL. The absorbance was determined with Victor Reader using a 540 nm filter.

2.9 Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed by means of ANOVA using Statgraphics package (STSC, Rockville, MD, USA). When significant differences were found, means were compared by Scheffè's F test. *p*-Values <0.05 were considered to be statistically significant.

3 Results

3.1 Granulosa cell viability

Resveratrol analogue **2** significantly ($p<0.05$) decreased ATP content only at the highest concentration tested, while analogue **3** displayed an inhibitory action at 1, 10, 100 μM ($p<0.001$) (Fig. 2).

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

MNFR 2009-00320 Journal Issue Number 1 Manuscript Number 2009-00320 Color Figure No. nil

UNCORRECTED PROOF

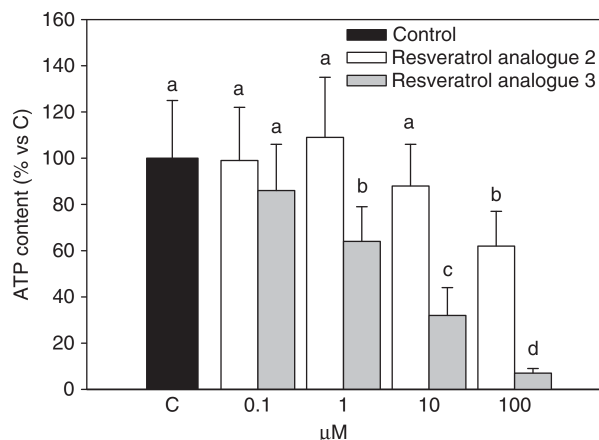


Figure 2. Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on granulosa cell proliferation using ATP content assay test. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p < 0.05$).

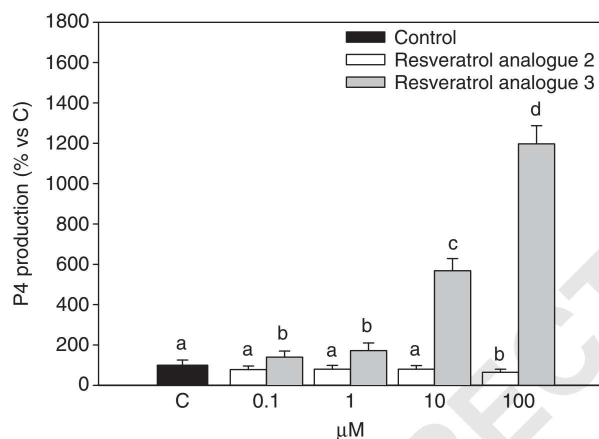


Figure 3. Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on P4 production in swine granulosa cell culture using RIA. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p < 0.05$).

3.2 Granulosa cell steroid production

P4 production (basal = 70 ± 9 ng/mL; means ± SEM) appeared significantly reduced only by the highest dosage of resveratrol analogue **2** ($p < 0.05$); on the contrary, resveratrol analogue **3** displayed a significant stimulatory effect on P4 levels at all concentrations tested, with a dose-dependent action at 10 and 100 μM ($p < 0.01$, Fig. 3).

As for E2 (3 ± 0.5 ng/mL), only 100 μM resveratrol analogue **2** enhanced the steroid production ($p < 0.01$), while resveratrol **3** was effective at all dosages in increasing E2 levels, with a dose-dependent action at 10 and 100 μM ($p < 0.001$, Fig. 4).

3.3 Granulosa cell VEGF production

Both reseveratrol analogues significantly reduced VEGF levels (900 ± 50 pg/mL) at 1, 10 and 100 μM ($p < 0.01$) without any significant differences among the concentrations tested ($p < 0.01$) (Fig. 5).

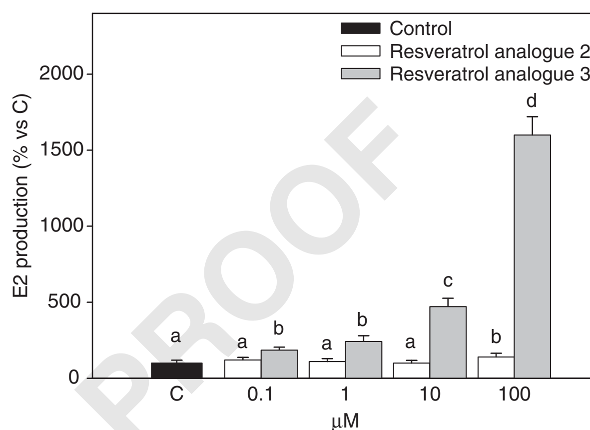


Figure 4. Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on E2 production in swine granulosa cell culture using RIA. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p < 0.01$).

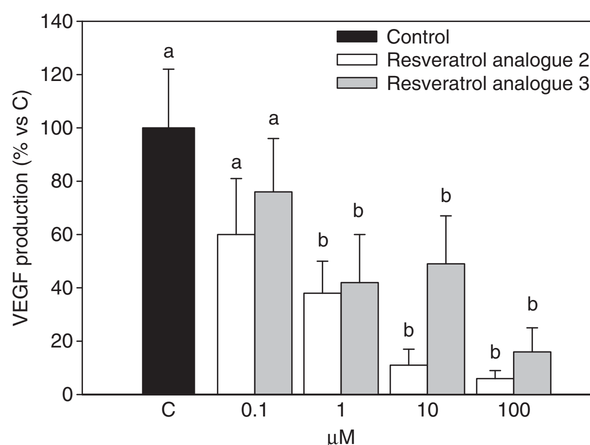


Figure 5. Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on VEGF production in swine granulosa cell culture using ELISA. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p < 0.01$).

UNCORRECTED PROOF
Colour in Web, Color in Print
Colour in Web, Color in Print
Colour in Web, Color in Print
Colour in Web, Color in Print

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

MNFR 2009-00320 Journal Issue Number 1 Manuscript Number 2009-00320 Color Figure No. nil

3.4 Granulosa cell redox status

Neither free radicals levels nor scavenging enzyme activity were significantly affected by resveratrol analogue **2** (Figs. 6–10). On the contrary, 10 and 100 μM resveratrol analogue **3** significantly ($p<0.001$) increased both free radicals production (Figs. 6 and 7) and at the same time potentiated ($p<0.001$) the activity of scavenging enzymes SOD (320 ± 10 mU/mL), peroxidase (1420 ± 220 mU/mL) and catalase (32 ± 7 mU/mL) (Figs. 8–10).

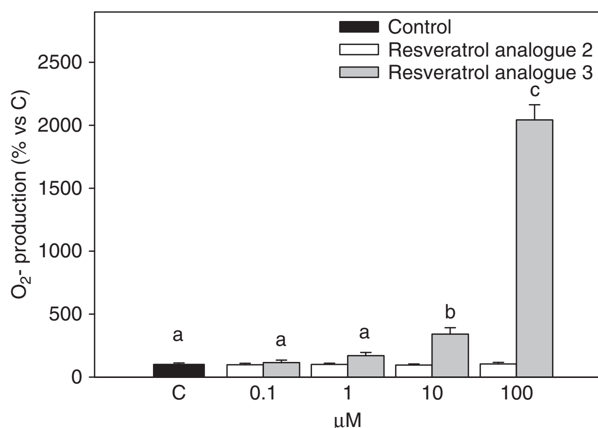


Figure 6. Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on O_2^- concentration in swine granulosa cell culture using WST-1 test. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p<0.05$).

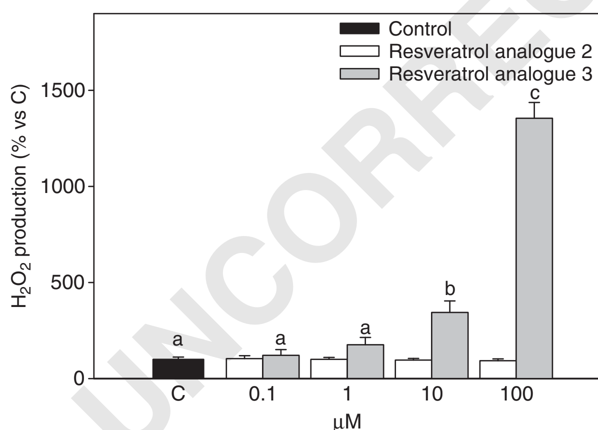


Figure 7. Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on H_2O_2 concentrations in swine granulosa cell culture using colorimetric method. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p<0.001$).

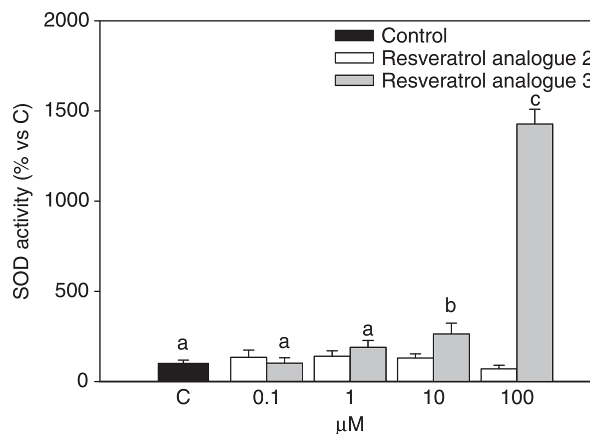


Figure 8. Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at the concentrations of 0.1, 1, 10, 100 μM on SOD activity in swine granulosa cell culture using colorimetric method. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters indicate a significant difference ($p<0.001$).

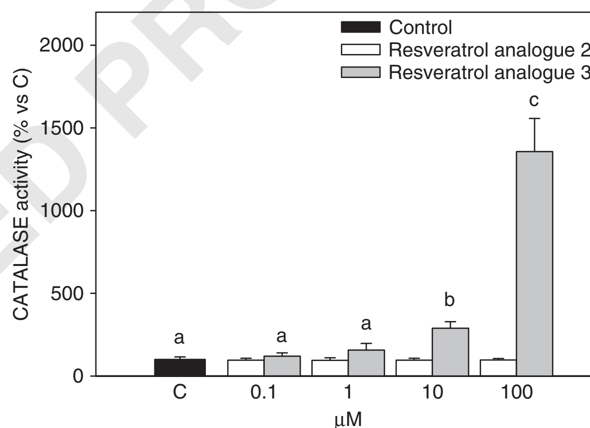


Figure 9. Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on Catalase activity in swine granulosa cell culture using colorimetric method. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p<0.001$).

4 Discussion

Resveratrol is a phytoalexin found in a wide variety of dietary sources including grapes berries and peanuts. A primary impetus for research on resveratrol was from the paradoxical observation that a low incidence of cardiovascular diseases may co-exist with a high-fat diet intake and moderate consumption of red wine, a phenomenon known as the French paradox. During the last years, this substance has been the focus of many *in vitro* and *in vivo* studies investi-

UNCORRECTED PROOF
Colour in Web, Color in Print

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

MNFR 2009-00320 Journal Issue Number 1 Manuscript Number 2009-00320 Color Figure No. nil

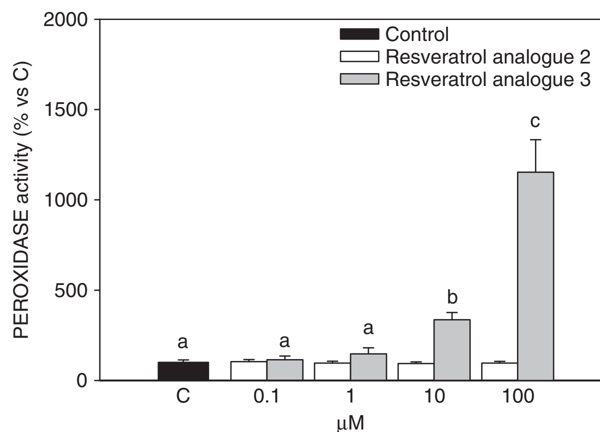


Figure 10. Effect of the 48 h treatment with or without (C) resveratrol analogue **2** or **3** at concentrations of 0.1, 1, 10, 100 μM on Peroxidase activity in swine granulosa cell culture using colorimetric method. Data, expressed as percentage *versus* respective C, represent the mean ± SEM of six replicates/treatment repeated in four different experiments. Different letters on the bars representing the same analogue indicate a significant difference ($p<0.001$).

gating its biological attributes, which include mainly antioxidant and anti-inflammatory activities, anti-platelet aggregation effect, anti-atherogenic properties and cancer chemoprevention. Some of these effects may be due in part to resveratrol being a phytoestrogen, with biological properties similar to those of estrogens [23]. However, resveratrol is characterized by low bioavailability [24], short half-life [25] and extensive conjugation [26] and consequently needs to be used in high doses for possible efficacy of therapeutic treatments. Thus, a number of synthetic analogues of resveratrol have already been synthesized and tested for their potential anticancer, anti-inflammatory and anti-angiogenetic effects [6, 9, 17]. Among the resveratrol analogues, polymethoxystilbenes represent a sub-group of great interest due to their higher bioavailability, at least in intestinal and colonic mucosae and in the brain, with respect to resveratrol [7]. Recently, the pharmacokinetics of 3,5,4′-trimethoxystilbene (**2**) has been studied [8]: in comparison with **1**, **2** showed greater plasma exposure, longer half-life and lower clearance. Thus, as a first step in a deeper evaluation of methylated analogues of resveratrol, we have recently [9] demonstrated the antiangiogenic effect of several polymethoxystilbenes; among these, the most effective appeared to be 3,5,4′-trimethoxystilbene and most of all its 2-hydroxy analogue. Therefore, these molecules were tested in the present study in order to unravel their biological action on different granulosa cell functions. Our overall results clearly show that the 2-hydroxy analogue appears the most potent compound in affecting cell functional parameters, except for VEGF production, which was inhibited similarly by both resveratrol analogues. This would suggest that the biological activity can be effectively modulated by a slight modification of the chemical structure: in particular, the improved effectiveness may be related to an increased diffusion rate in a hydrophilic medium or in a better interaction with a receptor/enzyme active site. In this regard, it is worth noting that subtle modifications in the structure of resveratrol analogues may noticeably affect their tubulin-inhibitory activity, due to the strict requirements for an effective interaction with the colchicine-binding pocket of tubulin [27]. Moreover, different biological effects related to the slight different structures of polihydroxylated stilbenoids have been reported [28].

In agreement with our previous paper [9] in which we documented a marked inhibition of endothelial cell growth, also granulosa cell viability was almost dose-dependently inhibited by the 2-hydroxy analogue. This feature could make this analogue an interesting compound for further evaluation as a potential cancer chemopreventive agent.

Data concerning the effects of the hydroxylated analogue **3** on steroidogenesis are remarkable, in that a potent stimulation of both P4 and E2 production was induced in granulosa cells. A similar effect was displayed by **2** on E2 production at the highest concentration tested. On the other hand, at the same dosage this analogue decreased P4 levels. At the moment the reason for these marked discrepancies are not clear. 3,5,4′-trimethoxystilbene biological action appears similar to that of resveratrol [29] on different steroid-producing cells; due to its stilbene structure, resveratrol has been related to the synthetic estrogen diethylstilbestrol and included into endocrine disrupting compounds, chemicals with the potential to elicit negative effects on endocrine system of human and animal [23].

As suggested above, we could presume that the hydroxylation of the analogue 3,5,4′-trimethoxystilbene has a significant impact on biological function: in particular, the hydroxyl group could act ahead of steroids biosynthetic pathway, thus stimulating both P4 and E2 production. Further studies will be necessary to verify this hypothesis.

The interplay between free radicals and antioxidant scavenging enzymes is important in maintaining health, and in determining the rate of aging and age-related diseases [30]. Free radicals induce oxidative stress, which is balanced by the body's endogenous antioxidant systems and by the ingestion of exogenous antioxidants. If the generation of free radicals exceeds the protective effects of antioxidants this can cause oxidative damage, which accumulates during the life cycle, and can be implicated in aging and age-dependent diseases such as cardiovascular disease, cancer, neurodegenerative disorders and other chronic conditions [31–33]. Recent results have provided interesting insight into the effect of resveratrol on intracellular redox status. These results seem to support both anti- and pro-oxidant activities of this compound [34]. As for polymethoxystilbenes, present data demonstrate that 3,5,4′-trimethoxystilbene does not affect in any way cellular redox status while the 2-hydroxy analogue stimulates the production of radical species and, most interestingly, potentiates the activity of all the scavenging enzymes. This ability may be caused by the

Colour in Web, Color in Print
UNCORRECTED PROOF
© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

MNFR 2009-00320 Journal Issue Number 1 Manuscript Number 2009-00320 Color Figure No. nil

presence of additional phenolic groups in its chemical structure as already suggested by Olas *et al.* [35] who evidenced a potent antioxidant effect by a naturally occurring resveratrol analogue *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene. In addition, Murias *et al.* [28] demonstrated that hydroxystilbenes with a different number of hydroxyl-groups and different substitution patterns exert different antioxidant potency.

Taken together, present data improve the knowledge about biological effects of polymethoxystilbenes and may be useful in order to develop resveratrol analogues for medical use.

This work was supported by MIUR, Ministero dell'Universit" e della Ricerca (PRIN 2007, Rome Italy), Universit" degli Studi di Catania (PRA, Catania, Italy) and Universit" degli Studi di Parma (FIL).

The authors have declared no conflict of interest.

5 References

[1] D'Archivio, M., Filasi, C., Di Benedetto, R., Gargiulo, R. *et al.*, Polyphenols, dietary sources and bioavailability. *Ann. Ist. Super. Sanità* 2007, *43*, 348–361.

[2] Nassiri-Asl, M., Hosseinzadeh, H., Review of the pharmacological effects of *Vitis vinifera* (Grape) and its bioactive compounds. *Phytother. Res.* 2009 doi: 10.1002/ptr.2761.

[3] Potter, G. A., Patterson, L. H., Wanogho, E., Perry, P. J., *et al.*, The cancer preventative agent resveratrol is converted to the anticancer agent piceatannol by the cytochrome P450 enzyme CYP1B1. *Br. J. Cancer* 2002, *86*, 774–778.

[4] Espín, J. C., García-Conesa, M. T., Tomás-Barberán, F. A., Nutraceuticals: facts and fiction. *Phytochemistry* 2007, *68*, 2986–3008.

[5] Fang, .J. G., Zhou, B., Structure-activity relationship and mechanism of the tocopherol-regenerating activity of resveratrol and its analogues. *J. Agric. Food Chem.* 2008, *56*, 11458–11463.

[6] Chillemi, R., Sciuto, S., Spatafora, C., Tringali, C., Anti-tumor properties of stilbene-based resveratrol analogues: recent results. *Nat. Prod. Commun.* 2007, *2*, 499–513.

[7] Sale, S., Verschoyle, R. D., Boocock, D., Jones, D. J. *et al.*, Pharmacokinetics in mice and growth-inhibitory properties of the putative cancer chemopreventive agent resveratrol and the synthetic analogue *trans* 3,4,5,4′-tetramethoxystilbene. *Br. J. Cancer* 2004, *90*, 736–744.

[8] Lin, H. S., Ho, P. C., A rapid HPLC method for the quantification of 3,5,4′-trimethoxy-*trans*-stilbene (TMS) in rat plasma and its application in pharmacokinetic study. *J. Pharm. Biomed. Anal.* 2009, *49*, 387–392

[9] Spatafora, C., Basini, G., Baioni, L., Grasselli, F. *et al.*, Antiangiogenic resveratrol analogues by mild *m*-CPBA aromatic hydroxylation of 3, 5-dimethoxystilbenes. *Nat. Prod. Commun.* 2009, *4*, 239–246.

[10] Basini, G., Bussolati, S., Santini, S. E., Bianchi, F. *et al.*, Hydroxyestrogens inhibit angiogenesis in swine ovarian follicles. *J. Endocrinol.* 2008, *199*, 127–135.

[11] Basini, G., Bianco, F., Grasselli, F., Tirelli, M. *et al.*, The effects of reduced oxygen tension on swine granulosa cell. *Regul. Pept.* 2004, *120*, 69–75.

[12] Basini, G., Grasselli, F., Bianco, F., Tirelli, M. *et al.*, Effect of reduced oxygen tension on reactive oxygen species production and activity of antioxidant enzymes in swine granulosa cells. *Biofactors* 2004, *20*, 61–69.

[13] Basini, G., Bianco, F., Grasselli, F., Epigallocatechin-3-gallate from green tea negatively affects swine granulosa cell function. *Domest. Anim. Endocrinol.* 2005, *28*, 243–256.

[14] Basini, G., Bussolati, S., Santini, S. E., Bianchi, F. *et al.*, Antiangiogenesis in swine ovarian follicle: a potential role for 2-methoxyestradiol. *Steroids* 2007, *72*, 660–665.

[15] Basini, G., Bussolati, S., Santini, S. E., Grasselli, F., Reactive oxygen species and anti-oxidant defences in swine follicular fluids. *Reprod. Fertil. Dev.* 2008, *20*, 269–274.

[16] Bianco, F., Basini, G., Grasselli, F., Angiogenic activity of swine granulosa cells: effects of hypoxia and vascular endothelial growth factor Trap R1R2, a VEGF blocker. *Domest. Anim. Endocrinol.* 2005, *28*, 308–319.

[17] Cardile, V., Chillemi, R., Lombardo, L., Sciuto, S. *et al.*, Antiproliferative activity of methylated analogues of E- and Z-resveratrol. *Z. Naturforsch. C* 2007, *62*, 189–195.

[18] Bachelor, F. W., Loman, A. A., Snowdon, L. R., Synthesis of pinosylvin and related heartwood stilbenes. *Can. J. Chem.* 1970, *48*, 1554–1557.

[19] Grasselli, F., Baratta, M., Tamanini, C., Effects of a GnRH analogue (buserelin) infused via osmotic minipumps on pituitary and ovarian activity of prepuberal heifers. *Anim. Reprod. Sci.* 1993, *32*, 153–161.

[20] Barboni, B., Turriani, M., Galeati, G., Spinaci, M. *et al.*, Vascular endothelial growth factor production in growing pig antral follicles. *Biol. Reprod.* 2000, *63*, 858–864.

[21] Benov, L., Fridovich, I., Is reduction of the sulfonated tetrazolium 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2-tetrazolium 5-carboxanilide a reliable measure of intracellular superoxide production?. *Anal. Biochem.* 2002, *310*, 186–190.

[22] Ukeda, H., Shimamura, T., Tsubouchi, M., Harada, Y. *et al.*, Spectrophotometric assay of superoxide anion formed in Maillard reaction based on highly water-soluble tetrazolium salt. *Anal. Sci.* 2002, *18*, 1151–1154.

[23] Hanet, N., Lancon, A., Del mas, D., Jannin, B. *et al.*, Effects of endocrine disruptors on genes associated with 17β-estradiol metabolism and excretion. *Steroids* 2008, *73*, 1242–1251.

[24] Wenzel, E., Somoza, V., Metabolism and bioavailability of *trans*-resveratrol. *Mol. Nutr. Food Res.* 2005, *49*, 472–481.

[25] Asensi, M., Medina, I., Ortega, A., Carretero, J., Inhibition of cancer growth by resveratrol is related to its low bioavailability. *Free Rad. Biol. Med.* 2002, *33*, 387–398.

[26] Walle, T., Hsieh, F., DeLegge, M. H., Oatis, J. E. *et al.*, High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab. Dispos.* 2004, *32*, 1377–1382.

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

UNCORRECTED PROOF

MNFR 2009-00320 Journal Issue Number 1 Manuscript Number 2009-00320 Color Figure No. nil

[27] Simoni, D., Invidiata, F. P., Eleopra, M., Marchetti, P. *et al.*, Design, synthesis and biological evaluation of novel stilbene-based antitumor agents. *Bioorg. Med. Chem.* 2009, *17*, 512–522.

[28] Murias, M., Jäger, W., Handler, N., Erker, T., *et al.*, Antioxidant, prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: structure-activity relationship. *Biochem. Pharmacol.* 2005, *69*, 903–912.

[29] Chen, Y. C., Nagpal, M. L., Stocco, D. M., Lin, T., Effects of genistein, resveratrol, and quercetin on steroidogenesis and proliferation of MA-10 mouse Leydig tumor cells. *J. Endocrinol.* 2007, *192*, 527–537.

[30] Lu, T., Finkel, T., Free radicals and senescence. *Exp. Cell. Res.* 2008, *314*, 1918–1922.

[31] Khandrika, L., Kumar, B., Koul, S., Maroni, P. *et al.*, Oxidative stress in prostate cancer. *Cancer Lett.* 2009 doi: 10.1016/j.canlet.2008.12.011.

[32] Zhang, H., Park, Y., Wu, J., Chen, X. *et al.*, Role of TNF-alpha in vascular dysfunction. *Clin. Sci. (Lond.)* 2009, *116*, 219–230.

[33] Aliev, G., Obrenovich, M. E., Reddy, V. P., Shenk, J. C. *et al.*, Antioxidant therapy in Alzheimer's disease: theory and practice. *Mini Rev. Med. Chem.* 2008, *8*, 1395–1406.

[34] Alarcon de la Lastra, C. A., Villegas, I., Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. *Biochem. Soc. Trans.* 2007, *35*, 1156–1160.

[35] Olas, B., Wachowicz, B., Nowak, P., Stochmal, A. *et al.*, Comparative studies of the antioxidant effects of a naturally occurring resveratrol analogue*trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene and resveratrolagainst oxidation and nitration of biomolecules in blood platelets. *Cell. Biol. Toxicol.* 2008, *24*, 331–340.

UNCORRECTED PROOF

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

MNFR 2009-00320 Journal Issue Number 1 Manuscript Number 2009-00320 Color Figure No. nil

ALLEGATO L

"Antiangiogenic properties of an unusual benzo [k.l] xantene lignan derived from CAPE (Caffeic acid Phenethyl Ester)"

Invest New Drugs. 2010. In stampa.

doi:10.1007/s10637-010-9550-z.

Invest New Drugs
DOI 10.1007/s10637-010-9550-z

PRECLINICAL STUDIES

Antiangiogenic properties of an unusual benzo[*k*,*l*]xanthene lignan derived from CAPE (Caffeic Acid Phenethyl Ester)

Giuseppina Basini · Laura Baioni · Simona Bussolati · Francesca Grasselli · Carmelo Daquino · Carmela Spatafora · Corrado Tringali

Received: 20 August 2010 / Accepted: 21 September 2010
© Springer Science+Business Media, LLC 2010

Summary Angiogenesis is normally a highly regulated process that occurs during development, reproduction, and wound repair. However, angiogenesis can also become a fundamental pathogenic process in cancer and several other diseases. To date, the synthesis of angiogenesis inhibitors has been researched in several ways also starting from bioactive plant compounds. In the present study, we tested both in an angiogenesis bioassay and in ovarian cell culture, the potential antiangiogenic effect of a natural-derived benzo[*k,l*]xanthene lignan (5). This unusual compound was synthesized through the biomimetic dimerization of CAPE (Caffeic Acid Phenetyl Ester), a bioactive component of honeybee propolis. The lignan showed a significant, dose-related inhibitory effect on new vessel growth in the angiogenesis bioassay and it inhibited Vascular Endothelial Growth Factor secretion in ovarian cell culture. Therefore, we indicate the natural-derived benzo[*k,l*]xanthene lignan 5 as a potential new angiogenesis inhibitor.

Keywords Angiogenesis · VEGF · Granulosa cells · Caffeic acid phenetyl ester

G. Basini (✉) · L. Baioni · S. Bussolati · F. Grasselli
Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti—Sezione di Fisiologia Veterinaria, Università degli Studi di Parma,
Via del Taglio 10,
43126 Parma, Italy
e-mail: basini@unipr.it

C. Daquino · C. Spatafora · C. Tringali
Dipartimento di Scienze Chimiche, Università degli Studi di Catania,
Viale A. Doria 6,
95125 Catania, Italy

Introduction

Angiogenesis, or new blood vessel growth is defined as a process in which a network of new blood vessels emerges from preexisting ones [1]. It has been shown that exponential tumor growth over a few mm in size is dependent on the recruitment of its own nutrition and oxygen supply by angiogenesis [2]. Since then, many studies have been set up for preventing, delaying or even completely eliminating cancer by suppressing its neovascularization [3]. Recent studies have provided evidence that a wide range of food-derived phytochemicals and other diet–associated compounds or their synthetic derivatives represent a cornucopia of potential new compounds acting as angiogenesis inhibitors and numerous bioactive plant compounds are tested for the potential clinical applications [4]. On this basis, we have recently carried out the biomimetic dimerization of caffeic acid esters with the aim to obtain new dimers related to previously known antiangiogenic lignans [5]. Methyl caffeate and CAPE (Caffeic acid phenethyl ester, 1), a bioactive component of honeybee propolis extract [6], were employed as substrates. Surprisingly, the dimerization through oxidative coupling mediated by manganese-based reagents afforded with good yield unusual benzo[*k,l*]xanthene lignans, related to a rare group of natural products [7]. In fact, only a few examples of benzo[*k,l*]xanthene lignans are reported in the literature, such as rufescidride (2) from *Cordia rufescens* [8], mongolicumin (3) from *Taraxacum mongolicum* [9], and yunnaneic acid H (4) from *Salvia yunnanensis* [10]. The biological properties of this group of lignans are completely unexplored up to date, due to their rarity in nature and unavailability from synthesis. Thus, we considered of some interest the evaluation of the CAPE dimer 5 (Fig. 1) as a possible new antiangiogenic agent, employing the angiogenesis bioassay set up in our laboratory [11].

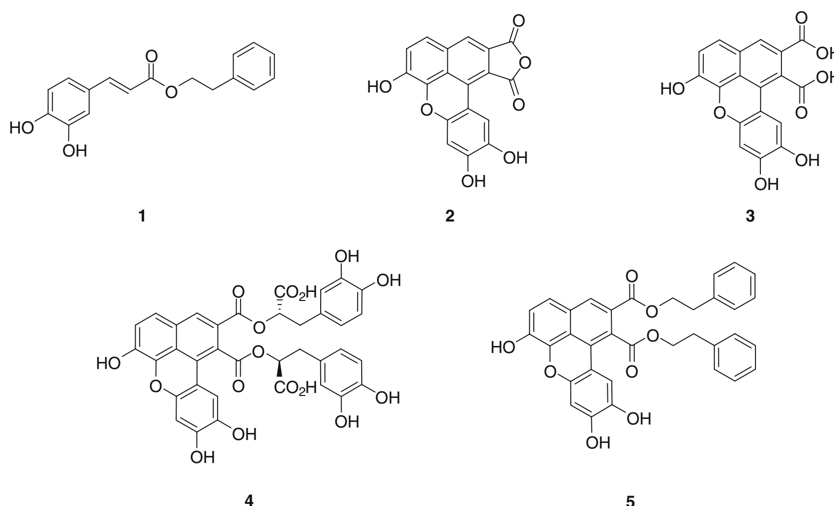


Fig. 1 Structure of compounds 1–5

Moreover, in order to better investigate the effect of the CAPE-derived lignan 5 on angiogenesis, swine ovarian granulosa cells were employed since they are actively involved in the regulation of follicular vascularization, a rare example of physiological angiogenesis [12, 13]. In addition, they are easy to recover. We used a previously well-defined experimental model [12, 14, 15] to study the effect of compound 5 on the production of Vascular Endothelial Growth Factor (VEGF), the main proangiogenetic peptide [16].

Materials and methods

Chemicals

All reagents used in this study were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

The compound 5 was synthesized according to a protocol previously reported by some of us [7] and based on oxidative coupling of CAPE (Caffeic Acid Phenethyl Ester) in the presence of $Mn(OAc)_3$ as oxidative agent.

Angiogenesis bioassay

Endothelial cell culture

An immortalized porcine aortic endothelial cell line (AOC) [17] was generously provided by José Yelamos (Hospital Universitario Virgen de la Arrixaca, El Palmar, 30120 Murcia, Spain). In all experiments, AOC at 19th passage were used and seeded in culture medium (CM) composed by M199 supplemented with sodium bicarbonate (2.2 mg/ml), bovine serum albumin (BSA 0.1%), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), selenium (5 ng/ml) and transferrin (5 μg/ml).

Three-dimensional endothelial cell culture on a fibrin gel support

The microcarrier-based fibrin gel angiogenesis assay was performed as described by Basini et al. [11]. Briefly, 12.5 mg gelatin-coated cytodex-3 microcarriers in 1 ml PBS were incubated for 3 h to hydrate. After two washings in PBS and one in CM, the microcarriers were put in flasks containg 5 ml CM; AOC (5×10^5) were added and cultured for 24 h in order to let the endothelial cells coat the microcarriers. For the fibrin gel preparation, 40 μl microcarriers covered by AOC were pipetted into 6 well plates containing a solution of fibrinogen (1 mg/ml PBS, pH 7.6), added with 1250 IU thrombine (250 μl). Fibrin gels were allowed to polymerize for 30 min at 37°C, then they were equilibrated for 60 min with 2 ml M199. After a change of

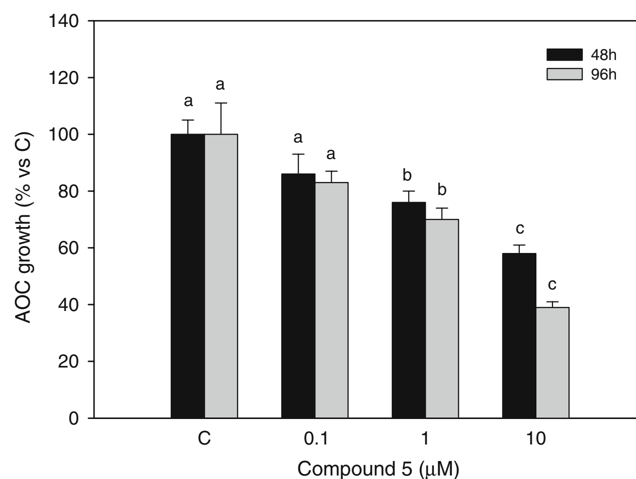


Fig. 2 Effect of the treatment with compound 5 (0.1, 1 or 10 μM) for 48 or 96 h on AOC growth in fibrin gel matrix. Different letters indicate a significant difference ($p<0.05$) among treatments in the same time as calculated by ANOVA and Scheffè' F test

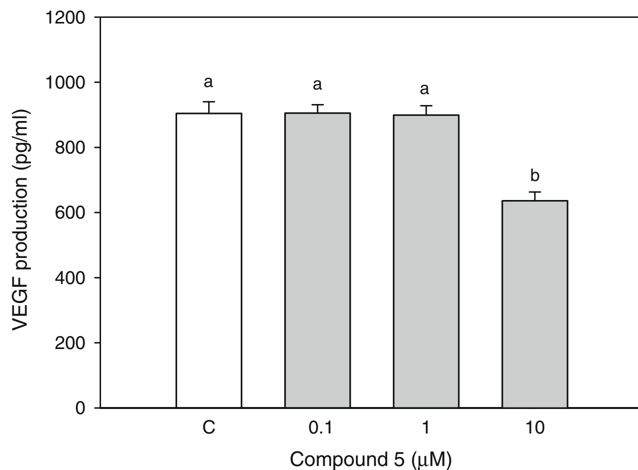


Fig. 3 Effect of the treatment with compound 5 (0.1, 1 or 10 μM) for 48 h on VEGF production by swine granulosa cell. Different letters indicate a significant difference ($p<0.001$) among treatments as calculated by ANOVA and Scheffè' F test

the medium, AOC were treated with VEGF (100 ng/ml; PeproTech EC Ltd, London, UK) in the presence or absence of 0.1, 1, 10 μM of 5. Plates were incubated at 37°C under humidified atmosphere (5% CO_2). AOC were cultured for 96 h, renewing totally the treatment after 48 h.

Quantification of AOC growth on fibrin gel matrix

Endothelial cell proliferation in the fibrin gel matrix was evaluated by means of the public domain NIH Program Scion Image Beta 4.02 (Scion Corporation, MA, USA, http://rsb.info.nih.gov/nih-image/). Ten pictures were taken for each gel at 48 and 96 h; images were converted into gray scale, resized to 50% (Paintbrush Software, MS Office) and saved as Bitmap 24bit format compatible with Scion. The modified images were then imported into the program and measurements were made drawing the perimeter of the area occupied by AOC expressed as number of pixel. In order to validate the measurement of the area covered by AOC in fibrin gels as a reliable method to evaluate cell proliferation, fibrin gels were stained by the nuclear dye bis-benzimide (Hoechst 33258, 20 μg/ml in PBS for 60 min) and examined by the fluorescence microscope [18]. This procedure was performed 20 times; for each experiment the number of nuclei was counted under fluorescence and pictures of the aera covered by AOC were taken in order to measure the surface covered in the fibrin gel. A strong correlation was observed between the area covered by AOC and the number of nuclei found in the same area ($r=0.96$).

Granulosa cell collection

Swine ovaries were collected at a local slaughterhouse from Large White cross-bred gilts, parity = 0. The stage of the cycle was unknown. Follicles were classified on a dimension-based fashion [15]. The ovaries were placed into cold phosphate buffered saline (PBS 4°C) supplemented with penicillin (500 IU/ml), streptomycin (500 μg/ml) and amphotericin B (3.75 μg/ml), maintained in a freezer bag and transported to the laboratory within 1 h. After a series of washings with PBS and ethanol (70%), granulosa cells were aseptically harvested by aspiration of large follicles (>5 mm) with a 26-gauge needle and released in medium containing heparin (50 IU/ml), centrifuged for pelleting and then treated with 0.9% prewarmed ammonium chloride at 37°C for 1 min to remove red blood cells. Cell number and viability were estimated using a haemocytometer under a phase contrast microscope after vital staining with trypan blue (0.4%) of an aliquot of the cell suspension. Cells were seeded in culture medium (CM) M199 supplemented with sodium bicarbonate (2.2 mg/ml), bovine serum albumin (BSA 0.1%), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), selenium (5 ng/ml) and transferrin (5 μg/ml). Once seeded, cells were incubated in the presence or absence of compounds 5 (0.1, 1 and 10 μM) and maintained for 48 h at 37°C under humidified atmosphere (5% CO_2). The treatment was identical for all experiments performed in this study.

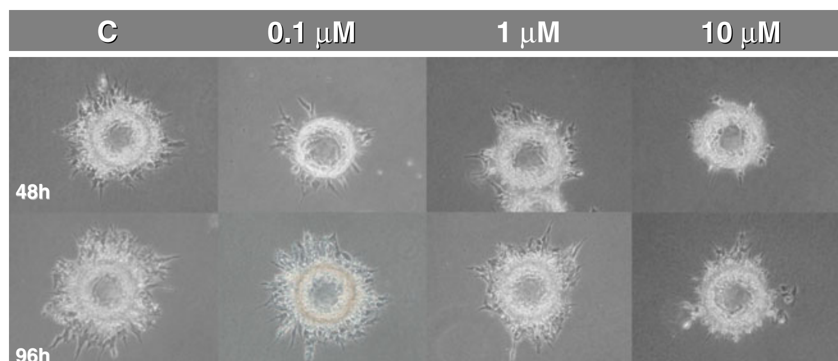


Photo 1 Phase contrast micrographs showing AOC growth at 48 and 96 h in fibrin gel matrix. Cells were cultured in CM or treated with compound 5 at the concentrations of 0.1, 1 or 10 μM

Granulosa cell VEGF production

10^6 granulosa cells in 1 ml CM+1% Fetal Calf Serum were seeded in 24-well plates and incubated for 48 h as indicated above. VEGF in culture media was quantified by an ELISA (Quantikine, R&D System, Minneapolis, MI, USA). This assay, developed for human VEGF detection, has been validated for pig VEGF [19]. The assay sensitivity was 8.74 pg/ml, the inter- and intra-assay coefficients of variation were always less than 7%. Victor Reader set to read at a wavelength of 450 nm emission was used to quantify the reaction product.

Statistical analysis

The experiments were repeated at least 4 times (4 replicates/treatment). Experimental data are presented as mean±SEM; statistical differences between treatments were calculated with Multifactorial ANOVA using Statgraphics package (STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by Scheffè's F test; p values<0.05 were considered to be statistically significant.

Results

Angiogenesis bioassay

Our data highlight that compound 5 exerts a dose-related inhibitory effect ($p<0.05$) on the angiogenic process at 1 and 10 μM, both after 48 and 96 h of incubation. On the contrary, the lowest concentration tested was always ineffective (Fig. 2; Photo 1).

Granulosa cell VEGF production

VEGF secretion by granulosa cells was significantly inhibited ($p<0.001$) by the highest concentration tested, while 0.1 and 1 μM were ineffective (Fig. 3).

Discussion

In most adult tissues, physiological angiogenesis is highly restricted and capillary growth occurs only rarely and in association with repair processes such wound and fracture healing. Disruption of the mechanisms controlling physiological angiogenesis has a major impact on health, as it underlies the pathogenesis of a growing list of diseases characterized by the overproliferation of blood vessels, including cancer, psoriasis, arthritis, retinopaties, obesity, asthma and atherosclerosis [20].

Phytochemicals have been shown to target and inhibit several key events of the angiogenic process such as proliferation and migration of endothelial cells as well as the expression of some pro-angiogenic factors [4]. Benzo[*k,l*]xanthene lignans, rarely encountered in nature, can be obtained by dimerization of CAPE (1), an honeybee propolis constituent with antioxidative, antiproliferative [6] and antiangiogenic properties [21–23]. The benzo[*k,l*]xanthene lignan 5, recently synthesized in our lab [7], represents an interesting compound to investigate due to its potential effects on angiogenesis. In this study, by means of an angiogenesis bioassay set up in our lab [11] we demonstrated that compound 5 displays a significant, dose-related inhibitory effect on new vessel growth at both concentrations tested. In addition, the CAPE analogue inhibited VEGF secretion by granulosa cells at 10 μM. Different components from propolis have been shown to possess high antiangiogenic activity: in particular, caffeic acid phenethyl ester, apigenin, artepillin C, galangin, kaempferol and pinocembrin are all effective in suppressing tube formation in HUVECS in a concentration-dependent manner [21]. These results have been confirmed by the observation that oral administration of propolis extract inhibits tumor-induced angiogenesis in vivo [24]. Other experimental evidences suggest that caffeic acid represents a promising anti-angiogenic agent which effectively inhibits retinal neovascularization without retinal toxicity [25].

To our knowledge, the effects of the synthethic derivatives of CAPE are still unexplored and this work represents the first evaluation of a benzo[*k,l*]xanthene lignan as an antiangiogenic agent. Thus, no structure-activity relationship can be established at the moment; however, our results suggest that benzo[*k,l*]xanthene lignans are worth of a deeper investigation which may open the way to the discovery of new natural-derived antiangiogenic agents.

Acknowledgements Supported by MIUR, Ministero dell'Università e della Ricerca (PRIN, Rome, Italy), Università degli Studi di Catania (PRA, Catania, Italy) and Università degli Studi di Parma (FIL, Parma, Italy).

References

1. Nussenbaum F, Herman IM (2010) Tumor angiogenesis: insights and innovations. J Oncol 132641. Epub 2010
2. Naumov GN, Akslen LA, Folkman J (2006) Role of angiogenesis in human tumor dormancy: animal models of the angiogenic switch. Cell Cycle 5:1779–1787
3. Murukesh N, Dive C, Jayson GC (2010) Biomarkers of angiogenesis and their role in the development of VEGF inhibitors. Br J Cancer 102:8–18
4. Varinska L, Mirossay L, Mojzisova G, Mojzis J (2010) Antiangiogenic effect of selected phytochemicals. Pharmazie 65:57–63

5. Apers S, Paper D, Buergermeister J, Baronikova S, Van Dyck S, Lemière G, Vlietinck A, Pieters L (2002) Antiangiogenic activity of synthetic dihydrobenzofuran lignans. J Nat Prod 65:718–720
6. Jaganathan SK, Mandal M (2009) Antiproliferative effects of honey and of its polyphenols: a review. J Biomed Biotechnol 2009:830616. Epub 2009
7. Daquino C, Rescifina A, Spatafora C, Tringali C (2009) Biomimetic synthesis of natural and 'unnatural' lignans and neolignans by oxidative coupling of caffeic esters. Eur J Org Chem 2009:6289–6300
8. Souza da Silva SA, Souto AL, de Fatima Agra M, Leitao da-Cunha E, Barbosa-Filho JM, Sobral da Silva M, Braz-Filho R (2004) A new arylnaphthalene type lignan from *Cordia rufescens* A. DC. (Boraginaceae). ARKIVOC 6:54–58
9. Shi S, Zhang Y, Huang K, Liu S, Zhao Y (2008) Application of preparative high-speed counter-current chromatography for separation and purification of lignans from *Taraxacum mongolicum*. Food Chem 108:402–406
10. Tanaka T, Nishimura A, Kouno Y, Nonaka G, Yang CR (1997) Four new caffeic acid metabolites, yunnaneic acids E - H, from Salvia yunnanensis. Chem Pharm Bull 45:1596–1600
11. Basini G, Bussolati S, Santini SE, Bianchi F, Careri M, Mangia A, Musci M, Grasselli F (2008) Hydroxyestrogens inhibit angiogenesis in swine ovarian follicles. J Endocrinol 199:127–135
12. Basini G, Bianco F, Grasselli F (2005) Epigallocatechin-3-gallate from green tea negatively affects swine granulosa cell function. Domest Anim Endocrinol 28:243–256
13. Basini G, Baioni L, Bussolati S, Grolli S, Kramer LH, Wagner GF, Grasselli F (2010) Expression and localization of stanniocalcin 1 in swine ovary. Gen Comp Endocrinol 166:404–408
14. Basini G, Bussolati S, Santini SE, Bianchi F, Careri M, Mangia A, Musci M, Grasselli F (2007) Antiangiogenesis in swine ovarian follicle: a potential role for 2-methoxyestradiol. Steroids 72:660–665
15. Basini G, Bussolati S, Santini SE, Grasselli F (2008) Reactive oxygen species and anti-oxidant defences in swine follicular fluids. Reprod Fertil Dev 20:269–274
16. Murukesh N, Dive C, Jayson GC (2010) Biomarkers of angiogenesis and their role in the development of VEGF inhibitors. Br J Cancer 102:8–18
17. Carrillo S, Chamorro M, Rodriguez-Gago B, Alvarez MJ, Molina MJ, Rodríguez-Barbosa JI, Sánchez A, Ramírez P, Muñoz A, Domínguez J, Parrilla P, Yélamos J (2002) Isolation and characterization of immortalized porcine aortic endothelial cell lines. Vet Immunol Immunopathol 89:91–98
18. Bianco F, Basini G, Grasselli F (2005) Angiogenic activity of swine granulosa cells: effects of hypoxia and vascular endothelial growth factor Trap R1R2, a VEGF blocker. Domest Anim Endocrinol 28:308–319
19. Barboni B, Turriani M, Galeati G, Spinaci M, Bacci ML, Forni M, Mattioli M (2000) Vascular endothelial growth factor production in growing pig antral follicles. Biol Reprod 63:858–864
20. Bhadada SV, Goyal BR, Patel MM (2010) Angiogenic targets for potential disorders. Fundam Clin Pharmacol 2010 Feb 22. [Epub ahead of print]
21. Ahn MR, Kunimasa K, Kumazawa S, Nakayama T, Kaji K, Uto Y, Hori H, Nagasawa H, Ohta T (2009) Correlation between antiangiogenic activity and antioxidant activity of various components from propolis. Mol Nutr Food Res 53:643–651
22. Izuta H, Shimazawa M, Tsuruma K, Araki Y, Mishima S, Hara H (2009) Bee products prevent VEGF-induced angiogenesis in human umbilical vein endothelial cells. BMC Complement Altern Med 17:9–45
23. Keshavarz M, Mostafaie A, Mansouri K, Shakiba Y, Motlagh HR (2009) Inhibition of corneal neovascularization with propolis extract. Arch Med Res 40:59–61
24. Ahn MR, Kunimasa K, Ohta T, Kumazawa S, Kamihira M, Kaji K, Uto Y, Hori H, Nagasawa H, Nakayama T (2007) Suppression of tumor-induced angiogenesis by Brazilian propolis: major components artepillin C inhibits in vitro tube formation and endothelial cell proliferation. Cancer Lett 252:235–243
25. Kim JH, Lee BJ, Kim JH, Yu YS, Kim KW (2009) Anti-angiogenic effect of caffeic acid on retinal neovascularization. Vascul Pharmacol 51(4):262–267