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Mesenchymal Stromal Cells Freeze-Dried Secretome (Lyo-secretome) for the treatment of musculoskeletal diseases in veterinary medicine.

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Abstract

In the last decade, regenerative medicine has gained interest in the veterinary clinical practice, since it offers a promising therapeutic alternative, especially for the treatment of musculoskeletal disorders (MSDs), characterized by multimodal management and complex healing processes. Among MSDs, osteoarthritis affects a high percentage of canine, equine, and even human populations, thus representing a one-health challenge. Furthermore, there is still a lack of effective long-term treatment for osteoarthritis. In this scenario, Mesenchymal stromal cells (MSCs) have been shown to modulate inflammation and stimulate the repair of damaged tissue, and they can have a positive long-term impact on the animal's quality of life. Despite promising results obtained in experimental and clinical studies, MSCs therapeutic applications are seriously influenced by the in-vivo implantation of an active and proliferating cell population. Recently, the new trend has been represented by MSCs-derived secretome, a cell-free product composed of soluble factors and extracellular vesicles, with similar biological activity but greater practical advantages when compared to the cells themselves. The present thesis work focused on a freeze-dried form of secretome, called Lyo-secretome, prepared from adipose-derived MSCs from canine and equine species. The first aim was the validation of the technological process for veterinary clinical-use medicinal production of different canine and equine batches of Lyo-secretome, and its characterization in terms of quantitative and qualitative analysis. Furthermore, *in vitro* metabolic tests were performed on target cells, such as tenocytes, chondrocytes, and synovial fluid derived-MSCs, demonstrating positive proliferative effects. Additionally, proteomic analysis demonstrated the presence of proteins involved in regulating the inflammatory response. The next step was a double-blinded randomized controlled clinical trial, enrolling dogs and horses suffering from naturally occurring osteoarthritis to assess the *in vivo* safety and efficacy of Lyo-secretome. The protocol included a double intra-articular injection of Lyo-secretome resuspended in hyaluronic acid, at 40 days interval. Clinical and orthopedic examinations, as well as owner questionnaires, were used to evaluate the patients during the 180-day follow-up. Results indicated that the double intra-articular injection of allogeneic Lyosecretome is safe and does not induce systemic adverse response for both species. A mild to moderate joint flare was observed in some patients after the injection, although they were self-limiting and not correlated to the treatment, since no differences were found with the control group (hyaluronic acid). Regarding the efficacy in canine patients, the study revealed mild clinical improvement following the two injections in both treatment and control

groups, without highlighting one treatment to be significantly superior, even if a positive tendency in terms of lameness and pain score, as well as an improvement in the Helsinki Chronic Pain Index score was observed in the Lyo-secretome group.

1. BACKGROUND

1.1 Mesenchymal Stromal Cells

Mesenchymal Stromal Cells (MSCs) are multipotent stem cells with self-renewal capability that can differentiate into tissues from the mesoderm. Since their isolation from the bone marrow by Friedenstein in the 1970s, MSCs have been isolated from a variety of both neonatal and adult tissues, being intensively investigated in clinical trials without the ethical issues associated with other types of stem cells, such as the embryonic ones [1]. MSCs sources include bone marrow, adipose tissue, umbilical cord, amniotic fluid, synovial tissues, liver, placenta, skin, and dental pulp. However, tissue from different sources exhibits different characteristics regarding differentiation potential, proliferative capacity, and total yield; furthermore, not all of them are equally suitable for clinical purposes [2]. Bone marrow was the first studied source, but low cell yields and the invasive aspiration procedure associated with elevated post-surgical pain have driven researchers to search for other sources. Currently, the most widely used source is adipose tissue, characterized by higher cell concentration and easier collection method, obtained by minimally invasive liposuction for the subcutaneous fat or during elective surgery for the visceral abdominal one [3]. Recently, perinatal tissues have also attracted increasing interest because of the relative simplicity of isolation and their lower immunogenicity, making them good candidates for clinical applications [4].

MSCs were defined as spindle-shaped cells able to adhere to plastic surfaces, according to the criteria developed by the International Society of Cell Therapy in 2006. At that time, they were characterized by a panel of markers genes, such as the expression of CD105, CD73, and CD90, but no expression of CD45, CD34, CD14 or CD11b, CD79a, CD19, or HLA-DR, and by the capacity to differentiate in osteoblasts, adipocytes, and chondroblasts *in-vitro* [5]. However, these criteria need to be revised to define MSCs, as variations exist at multiple levels. The heterogeneity of MSCs is determined by multiple factors, including but not limited to donors and tissue sources, cell populations, culture conditions, cell isolation techniques, and cryoprotective and thawing protocols [1].

In terms of donors, distinct functions can be due to differences in age, sex, breed, health condition, and other individual characteristics [6]. Based on the donor and their clinical use, MSCs can be classified into three classes: autologous, allogeneic, and xenogeneic. Since they are harvested from the patient's body, the most crucial advantage of autologous MSCs is the lack of immunogenic reaction following clinical application. Furthermore, their use does not imply ethical issues as the

donor corresponds to the receiving animal [7]. On the downside, the collection procedure can be invasive, especially in old or immunodeficient animals. In some cases, autologous therapy must be excluded a priori because of too high anesthesiologic risk. Further, the MSCs' in vitro expansion can be quite unpredictable as it strictly depends from patient to patient, and the time taken for culture and processing the therapeutic dose of MSCs can represent a deterrent when a quick clinical treatment is needed [8]. A clear recent trend is to shift from using autologous to "ready-to-treat" allogeneic or xenogeneic MSCs, which means that they can be prepared and stored in commercial quantities. Allogeneic MSCs are derived from a donor animal of the same species as the receiving animal, while the application of donor MSCs of a different species is called xenogeneic. The possibility of allogeneic or xenogeneic MSCs therapy was studied in several canine and equine studies, which described MSCs as "immune privileged" as a consequence of an absent major histocompatibility complex (MHC) class II expression on the cell surface, responsible for initiating an antigen-specific immune response interacting with CD4⁺ T cells [7]. However, recent studies suggest that MHC II could be up-regulated in vitro when MSCs are exposed to inflammatory environments, making this topic still a matter of debate [3]. An increasing number of veterinary MSCs-based products prepared from allogeneic or xenogeneic tissue have recently been available in commerce. In this case, a crucial aspect is that MSCs must be previously screened and tested for the absence of species-specific transferable pathogens [9].

The idea behind the therapeutic clinical application of MSCs is that in case of tissue damage, endogenous MSCs are naturally released into circulation and migrate to the site of injury, promoting regeneration [10]. The same mechanism, called "homing", is shown by transplanted MSCs that preferentially home targeted tissues guided by a complex multistep process to exit circulation and migrate to the injury site via a chemokine gradient involving several cytokines, chemokines and adhesion molecules [11]. Initially, it was thought that MSCs exerted their therapeutic effects upon reaching the affected site, actively replicating and differentiating to regenerate the tissue. Nowadays, it is largely demonstrated that MSCs act instead as a "drug store" by secreting a variety of biological molecules, called together secretome, with significant pro-regenerative, immunomodulating, angiogenic and anti-apoptotic effects [12].

1.2 MSCs-derived secretome

The term secretome refers to a complex mixture of different bioactive molecules released in the culture-conditioned medium (CM) in-vitro or in the extracellular space in-vivo, including soluble factors such as growth factors, cytokines, chemokines, and extracellular matrix components, and insoluble factors called extracellular vesicles (EVs) composed by exosomes, microvesicles and apoptotic bodies [13].

MSCs and their secretome have strong immunomodulatory capabilities, regulating the function of various immune cells, especially monocytes/macrophages, T-cells, natural killer cells and B-cells [14-17]. Similar to MSCs, the composition of their derived secretome changes depending on the existing stimuli in the microenvironment at the damaged tissue site or in vitro under different culture conditions [18]. MSCs-CM indeed contains several anti-inflammatory cytokines such as TGF β 1, IL-13, IL18BP (IL18 binding protein), CNTF (ciliary neurotrophic factor), NT-3 (neurotrophin 3 factor), or others like IL-10, IL-12p70, IL-17E, IL-27 and IL-1Ra; however, also pro-inflammatory factors like IL-6, IL-8, IL-9 or IL-1b have been found. The balance between these two components determines the final biological effects at the tissue site [19]. Pretreatment of MSCs with different in vitro stress, such as hypoxia, inflammatory factors, or pharmaceutical drugs or with different culture conditions, such as 3D culture or engineering methods, or a combination of these methods prior to application is a novel strategy to modulate the MSCs secretome composition, thus its final therapeutic activity [20]. MSCs treated with TNF- α and IFN- γ , for example, produced exosomes with increased immunosuppressive properties, with higher levels of IL-10 and PGE2, both involved in regulating the inflammatory process [21]. Inflammatory and hypoxic conditions also regulate the concentration of pro- or anti-angiogenic factors in MSCs-CM; hypoxia-inducible factor 1 alpha (HIF-1 α), for example, induces increased production of vascular endothelial cell growth factor (VEGF) and has a crucially important role for pro-angiogenic effects of VEGF-containing MSCs-Exosome [22]. MSCs-derived secretome was also able to regulate apoptosis in physiological and pathological conditions. MSCs-CM-based therapy significantly decreased the expression of pro-apoptotic Bax and cleaved caspase-3 but increased the expression of anti-apoptotic Bcl-2 in parenchymal cells, preventing their loss during ongoing inflammation [19].

A large number of immunomodulatory and growth factors are enveloped by a bilayer membrane; together with genetic materials, enzymes, signalling and signal transduction proteins, they represent the EVs content. Among EVs, exosomes are the cell-free therapeutic agent most

investigated in the literature. Due to their small size, they are easily carried to distant sites via biological fluids and released into the extracellular space, where they exert their biological effects by modulating multiple cell signalling pathways [23]. MSC-EVs can interact with tissue-specific target cells, binding to membrane-bound receptors and triggering intracellular signalling, which enables internalization of their content in the target cells, or, alternatively, EVs may fuse with the plasma membrane and deliver their content to the cytosol of the target cell directly [24,25].

Both EVs and soluble fraction of MSCs-derived secretome promote tissue regeneration and neo-angiogenesis and modulate immune response, thus representing promising strategies for the treatment of immune-mediated and inflammatory disorders [24]. Furthermore, MSCs secretome, as a cell-free therapy, offers several practical advantages, bypassing many limitations of MSCs-based therapy, including uncontrolled differentiation, unwanted immune reactions, and tumorigenic risk. MSCs secretome can be massively produced from a characterized cell type; it is easier to handle and store, and ready to use for the treatment of acute conditions, avoiding the time-consuming cell culture expansion. Additionally, MSCs' secretome content can be quantitatively and qualitatively characterized as any conventional pharmaceutical agent [19,23,26].

In laboratory practice, several methods exist to produce and isolate MSCs' secretome.

Once centrifuged to remove cell debris, the conditioned medium can be collected and contains soluble and insoluble fractions of the secretome. The CM can be further purified using different techniques; ultracentrifugation is considered the gold standard to isolate EVs. However, it is time-consuming and is limited by the risk of EVs disruption or aggregation. Size-based isolation techniques such as ultrafiltration, tangential flow filtration or size exclusion chromatography have turned out to be interesting strategies and can be combined with ultracentrifugation to isolate and purify the EVs [27,28]. It is well known that the isolation method indeed affects the amount, type and purity of EVs and their biological effect. Despite the advances, there is still a need for scalable isolation techniques that present a high yield, maintaining the characteristics of the final products [29].

Based on this scenario, in 2018, Bari and colleagues presented an innovative GMP (Good Manufacturing Practice) compliant method for the large-scale production of a freeze-dried form of secretome (Lyo-secretome) derived from human adipose tissue MSCs [30]. In this pilot study, ultrafiltration and freeze-drying techniques were combined, and a quality control panel, including physicochemical and molecular characterization, was performed on the final powder. Moreover,

sterility tests, cytotoxicity, and blood compatibility assay were assessed for safety requirements. A deeper characterization was also performed, checking total protein and lipid content, exosome yields and proteomic analysis; interestingly, proteins involved in immune and oxidative stress response were among the most represented. The final aim was to find a scalable and GMP-compliant production process that would allow standardized products suitable for clinical applications to be obtained [30,31].

MSCs-derived secretome indeed has been considered a new, cell-free therapeutic approach for treating several inflammatory and degenerative diseases, such as osteoarthritis (OA). In-vitro evidence suggested the possible chondroprotective activity of MSCs-secretome, such as the study of Hotham and colleagues who tested EVs isolated from bone marrow MSCs on artificially-inflamed equine chondrocytes, demonstrating the up-taken into the targeted cells and anti-inflammatory effects on gene expression [32], or again Chen et al. showed that MSCs-CM-treated chondrocytes significantly reduced production of inflammatory cytokines (TNF- α , IL-1 β , IL-6), which play detrimental role in cartilage degeneration during OA development and progression [33]. Even more, it was demonstrated that CM and exosomes from adipose tissue-derived MSCs enhanced the production of immunosuppressive IL-10 in IL-1 β -activated OA chondrocytes, indicating their anti-inflammatory and chondroprotective effects [34]. The therapeutic potential of MSCs-secretome was confirmed in preclinical in vivo studies, where exosomes intra-articular injection was shown to have both protective and therapeutic effects in the damaged joints of OA murine models [35,36]. Indeed, it has been observed that MSC-derived CM and isolated EVs were able to ameliorate OA symptoms by preventing joint deterioration and improving tissue remodelling in mice joint disease models [37]. Despite these promising findings, very limited data is available on the safety and efficacy of cell-free therapy using MSCs in veterinary patients, and clinical controlled trials are required to establish the therapeutic efficacy of these therapies in naturally occurring musculoskeletal disorders.

1.3 Clinical trials using MSCs and derived secretome to treat osteoarthritis in companion animals

Musculoskeletal disorders in veterinary medicine: a focus on osteoarthritis

Among the different species, dogs and horses are the most deeply investigated in regenerative veterinary medicine, and among the different diseases, musculoskeletal disorders (MSDs) are commonly found in the veterinary practice and represent the most studied in this therapeutic approach. The first evidence of MSCs-based therapy in vivo involved reimplanting equine autologous BM-MSCs into a central lesion of the superficial digital flexor tendon (SDFT). This study highlighted the feasibility of using cultured expanded MSCs for therapeutic purposes and the safety of their injection [38]. Since then, MSCs have been successfully used for both dogs and horses for the treatment of MSDs, as well as for cartilage damage. Among MSDs, joint problems such as osteoarthritis (OA) rank first, representing 60% of lameness in horses and, in most cases, leading to a sudden end of the sportive career of racehorses [39]. OA also has a high prevalence in the canine population, affecting 20% of patients in North America [40]. OA is a chronic degenerative joint disease and represents a “one-health” challenge as it is one of the leading causes of disability in human patients worldwide.

Furthermore, a close analogy between human and veterinary patients makes dogs and horses useful OA clinical models [41,42]. The mechanisms of this complex disease involve cartilage, subchondral bone, synovium, tendons, ligaments, muscles, and even neural tissues. It is characterized by inflammation, causing cartilage degradation, remodelling of subchondral bone, as well as severe clinical symptoms such as joint swelling, pain, stiffness, and difficulty moving the affected joint. In the late stage, OA becomes a systemic disease [43,44]. The two main categories of OA are mechanical when healthy articular cartilage undergoes excessive loads leading to degeneration, and structural, when articular cartilage is weak, with minor defects or other abnormalities contributing to rapid degradation. Secondary OA might result from previous tendon or ligament injury, joint instability due to intra-articular fracture, or wear and tear of the articular cartilage [43].

Currently, there is no cure for OA. The conventional treatments focus on pain relief and reduction of symptoms through the use of anti-inflammatory or analgesic drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids or opioids, associated with physical therapy, diet, weight management, and dietary supplements, with surgical treatments being applied in severe cases [9,40]. OA drug delivery approaches, focusing on lubrication-based biomaterials and drug delivery systems, represent recent advantages in the management of OA [39]. The greatest

challenge with conventional OA treatments is that they do not stop the disease but only focus on damage reduction and symptom management. The use of MSCs-based therapies for OA has shown promising pre-clinical and clinical results in both canine and equine patients, suggesting a possible therapeutic role of MSCs in the treatment of joint disorders [45].

MSCs-based therapy for canine naturally occurring osteoarthritis

To better understand the therapeutic potential of MSCs-based therapies, using naturally occurring diseases instead of model-based studies is closer to reality, especially in musculoskeletal disorders, characterized by a complex healing process [45]. Other positive aspects are the preservation of experimental animals, the therapeutic opportunity, and long-term follow-up for the affected companion animals. Furthermore, spontaneous disorders represent a suitable “one-health” model since the pathophysiology is very similar between canine, equine, and human species.

On the downside, owned patient recruitment represents a big challenge; the study populations are often too small, and an actual control group is frequently missing because owners want their pets to be treated. Moreover, OA-affected dogs often have problems in multiple joints and the severity of OA can differ, which may complicate study outcome interpretation [7].

In general, studies evaluating canine naturally occurring OA presented recurrent inclusion criteria, defined as the presence of clinical signs of chronic OA, with pain and lameness, non-responsive to conventional treatments in one or more joints. Radiography was used to confirm OA diagnosis and starting grade. Furthermore, patients should suspend any kind of therapy, especially glucocorticoids or NSAIDs, before enrollment and for the entire study period, when compatible with the clinical state, to avoid any confounding element. Exclusion criteria usually were the presence of severe systemic diseases evaluated with a complete blood check or clinical signs consistent with other orthopaedic or neurological diseases [46–78]. In some studies, body weight was used as a selective parameter, as it should be above or below a given number or strictly stable for the entire duration of the protocol [47–49,51,54,59,71,72]. No limitations in terms of sex were imposed, while breed or age in some cases were selected a priori [46,49,51,53,54,60].

Autologous cells have been the first type of MSCs investigated in the treatment of spontaneous canine osteoarthritis, starting with the pioneer clinical trials of Black and colleagues in 2007 and 2008 [64,65]. The use of autologous adipose-derived MSCs, administered with a single intra-articular injection followed by clinical, orthopaedic, and owner evaluations will be the baseline

protocol repeated in many other subsequent papers, with a therapeutic dose ranging from 1 to 5×10^6 of MSCs [64–67,72,73,76]. Once the safety (and the efficacy) of these injections were demonstrated, in the following years, higher numbers of cells were administered intra-articularly, ranging from 4 to 30×10^6 per joint, showing similar positive outcomes presented in the previous studies [68–71,74,75]. In the work of Muir and colleagues, bone marrow is used as an alternative source of autologous MSCs administered with the combination of endovenous (2×10^6 /kg of body weight) and intra-articular (5×10^6) applications [76]. In some cases, supportive products such as Hyaluronic Acid (HA) or Platelet Rich Plasma (PRP) can be combined with MSCs for the management of osteoarthritis [66,67,70,75,77]. Even if the most recent studies have been focusing on allogeneic or xenogeneic products as a preferred future therapeutic direction, a 2023 retrospective study by Armitage and colleagues investigated the use of autologous MSCs in 245 dogs unresponsive to conventional treatment, demonstrating that autologous MSCs remain a targeted, holistic approach in the “real world” for the treatment of musculoskeletal pathology [77]. Autologous studies details are resumed in **Table 1**.

In allogeneic treatments, adipose tissue is confirmed to be the most used source of MSCs isolation [52,54–57,59–62], but also dental pulp and fetal tissue-derived MSCs have been investigated [53,58,63]. MSCs can be administered with a single [52,54–57,60] or multiple [53,58,59,61–63] intra-articular injection, but the amount of cells is once again very variable between these publications, ranging from 2×10^5 to 12×10^6 . No significant side effects were observed in the majority of the papers, except for very few cases of self-limiting local joint flare, usually restored in 24-48h [55–58,62]. Since MSCs are known to have homing capacities, some studies investigated other administration routes, such as endovenous infusion or acupuncture [52,57,59]. Also, in allogeneic studies, in some cases, MSCs can be associated [55,60] or compared [61,62] with HA or PRP. Allogeneic studies details are resumed in **Table 2**.

Author	# Patients	Group/treatment	Joint	Donor/source	Injection route	Dose	# Injections	Evaluations	Side effects	Outcome	Follow-up
Black et al., [65]	21	MSCs and Control not specified	hip	adipose tissue	IA	4,2-5x10 ⁶	1	clinical and orthopedic parameters	NO	positive	180 days
Black et al., [64]	14	MSCs:14	elbow	adipose tissue	IA	3-5x10 ⁶	1	clinical and orthopedic parameters	NO	positive	90 days
Guercio et al., [66]	4	MSCs+HA: 2 MSCs+PRP: 2	elbow	adipose tissue	IA	3-5x10 ⁶	1	clinical, orthopedic, and radiological parameters; owner evaluation	NO	positive	1 month
Yoon et al., [67]	1	MSCs+surgery+HA: 1	stifle	adipose tissue	IA	1x10 ⁶	1	clinical, orthopedic and radiological parameters	NO	positive	19 months; long-term: 5 years
Vilar et al., [68]	13	MSCs+PRP:8 Control: 5	hip	adipose tissue	IA	15x10 ⁶	1	clinical, orthopedic, radiological parameters, and owner evaluation	NO	positive	6 months
Cuervo et al., [71]	35	MSCs: 18 PRP: 17	hip	adipose tissue	IA	30x10 ⁶	1	clinical and orthopedic parameters and owner evaluation	NO	positive	6 months
Nicpon et al., [72]	12	MSCs: 8 Control: 4	elbow	adipose tissue	IA	1,5x10 ⁶	1	clinical, orthopedic and synovial fluid evaluations	NO	positive	180 days
Vilar et al., [70]	15	MSCs: 10 Control: 5	hip	adipose tissue	IA	15x10 ⁶	1	clinical, orthopedic and gait analysis	YES (1 dog)	positive	3 months
Mohoric et al., [73]	10 (20 stifles)	MSCs: 10 Control: 10	stifle	adipose tissue	IA	2-3x10 ⁶	1	clinical, orthopedic and radiological parameters; synovial fluid analysis	NO	positive	12 months
Vilar et al., [69]	15	MSCs: 10 Control: 5	hip	adipose tissue	IA	15x10 ⁶	1	clinical, orthopedic and gait analysis	NO	positive	6 months
Muir et al., [76]	12	MSCs: 12	stifle	bone-marrow	IV+IA	MSCs/kg IV and 5x10 ⁶ IA	1	radiological parameters, blood and synovial fluid analysis	NO	positive	8 weeks
Szrentic Drazilov et al., [74]	10	MSCs: 10	different joints	adipose tissue	IA	>15x10 ⁶	1	clinical, orthopedic and radiological parameters	NO	positive	90 days; long-term: 4 years (5 dogs)
Sanghani et al., [75]	28 joints	MSCs+PRP: 28	different joints	adipose tissue	IA	depending on the body weight	1	clinical, orthopedic and gait analysis, owner evaluation	NO	positive	24 weeks
Armitage et al., [77]	245 (retrospective study)	MSCs alone or with PRP	musculoskeletal disorders	adipose tissue	different routes	different doses	ranging from 1 to 8	Objective outcome measures, validated subjective outcome measures	NO	positive	104 weeks

Table 1. Presentation of studies on naturally occurring OA applying autologous MSCs in dogs.

Author	# Patients	Group/treatment	Joint	Donor/source	Injection route	Dose	# Injections	Evaluations	Side effects	Outcome	Follow-up
Marx et al., [52]	9	autologous SVF: 4 allogeneic MSCs: 5	hip	adipose tissue	acupuncture	2 to 5 × 10 ⁶ (aSVF); 2 to 8 × 10 ⁵	1	clinical and orthopaedic examinations	NO	positive (especially in SVF group)	1 month
Booth et al., [53]	8	MSCs: 8	hip	dental pulp	IA	5 × 10 ⁶	2	clinical and orthopaedic examinations and owner questionnaire	NO	positive	4 month
Harmann et al., [54]	74	MSCs: 38 Control: 36	different joints	adipose tissue	IA	12 × 10 ⁶	1	CSOM, veterinary and owner assessments clinical and orthopaedic examinations and owner questionnaire, arthroscopy (n=1)	NO	positive	2 months
Kriston-Palet al., [55]	30	MSCs in HA: 30	elbow	adipose tissue	IA	12 × 10 ⁶ ± 3.2 × 10 ⁶	1	examinations and owner questionnaire, arthroscopy	YES (2 of 39)	positive	1 year
Shah et al., [57]	203	MSCs IA: 128 MSCs IV: 65	different joints	adipose tissue	IA or/and IV	not specified	1	clinical and orthopaedic examinations	YES (2 dogs) MSC: 5 <48h	90% YES IA; 76% YES IV	10 weeks
Cabon et al., [58]	22	MSCs: 22	different joints	fetal adnexa	IA	> 10 × 10 ⁶	1 (8 patients repeated the IA)	clinical evaluation and owner assessment	Repeated treatment: 4 <48h	positive	6 months to 2 years
Olsen et al., [59]	13	MSCs: 13	elbow	adipose tissue	IV	1–2 × 10 ⁶ /kg body weight	3	gait analysis, accelerometry, owner questionnaires, joint fluid analysis	NO	positive (owner) no difference (objective)	6 months
Wits et al., [60]	12	MSCs: 4 MSCs+HA: 4 Control: 4	hip	adipose tissue	IA	5 × 10 ⁶	1	clinical, orthopaedic and radiological parameters	NO	no difference	3 months
Kriston-Palet al., [56]	58	MSCs: 58	different joints	adipose tissue	IA	12 × 10 ⁶ ± 3.2 × 10 ⁶	1	owners' subjective observations clinical and orthopaedic examinations, gait analysis and owner questionnaire	YES (2 local inflammation)	positive	4–5 years
Okamoto-Okubo et al., [61]	16	MSCs: 8 PRP: 8	hip	adipose tissue	IA	5 × 10 ⁵	2	examinations, gait analysis and owner questionnaire objective gait analysis, accelerometry, clinical metrology instruments and veterinary exams, owner questionnaire	NO	positive	2 months
Kim et al., [62]	14	MSCs: 9 HA: 5	elbow, hip	adipose tissue	IA	10 × 10 ⁶	2	examinations, gait analysis, accelerometry, clinical metrology instruments and veterinary exams, owner questionnaire	YES (Self-limiting joint flare in 7 patients treated with MSCs)	negative	48 weeks
Domaniza et al., [63]	22 (elbows)	MSCs: 13 elbows Control: 9 elbows	elbow	Amnion	IA	approximately 2 × 10 ⁶	2	Kinematic gait analysis, synovial fluid biomarkers,	NO	positive	28 days

Table 2. Presentation of studies on naturally occurring OA applying allogeneic MSCs in dogs.

In xenogeneic naturally occurring studies, a pilot paper published in 2014 investigated the intra-articular transplantation of 5×10^6 porcine adipose-derived MSCs in three patients affected by stifle OA. Injection of porcine Ad-MSCs was safe and associated with most patients' orthopaedic and owner evaluation improvements [47]. Recently, the leading xenogeneic donor seems to be the equine species, given that the four articles cited in the literature tested the use of equine peripheral blood-derived MSCs [48,50,78] or equine umbilical cord-derived MSCs [49]. Equine MSCs were administered intra-articularly [47–49] or endovenously [50,78], both demonstrating positive outcomes and the absence of adverse response. Interestingly, for both administration routes, a wide array of significantly different doses was used (1 to $7,5 \times 10^6$ IA; 0,3 to 1×10^6 IV), highlighting how far the distance is towards standardized protocols. Xenogeneic studies details are resumed in **Table 3**.

Author	# Patients	Group/treatment	Joint	Donor/source	Injection route	Dose	# Injections	Evaluations	Side effects	Outcome	Follow-up
Tsai et al., [47]	3	MSCs: 3	stifle	porcine fat	IA	5×10^6	1	orthopedic assessment, owner questionnaire, X-ray imaging, and force-plate gait analysis.	NO	positive	12 weeks
Daems et al., [48]	6	MSC: 6 Control: 6 (same dogs, 6 weeks before MSC)	elbow	equine PB-MSCs	IA	1×10^6	1	orthopedic/clinical examination, gait analysis, hematological and synovial fluid analysis, RX, owner questionnaire	NO	positive (owner) no difference (objective)	12 weeks
Punzón et al., [49]	80	MSCs: 40 Placebo: 40	elbow, hip	equine UC-MSCs	IA	$7,5 \times 10^6$	1	gait analysis, orthopedic assessment and owner questionnaire	NO	positive (best response after 8 weeks)	12 weeks + 18 months
Beerts et al., [78]	1	MSCs: 1	stifle	equine PB-MSCs	IV	1×10^6	1	body scintigraphy imaging technique	NO	/	6h
Brondeel et al., [50]	50	MSCs: 50	elbow, stifle, hip	equine PB-MSCs	IV	3×10^5	1	clinical and orthopaedic examinations, CBPI	NO	positive	6 weeks

Table 3. Presentation of studies on naturally occurring OA applying xenogeneic MSCs in dogs.

At the time of writing, only two published research studies focused on the use of MSCs-derived cell-free products to manage naturally occurring canine osteoarthritis, indicating the need for a deeper investigation in this field. In 2020, Hunakova and colleagues studied the intra-articular injection of allogeneic Ad-MSCs derived conditioned medium in Labrador retriever dogs suffering from bilateral elbow OA, assessing synovial fluid markers and kinematic analysis of ROM for up to 42 days, demonstrating no side effects and the presence of positive outcome [51]. The second study was conducted by our group in 2021 to investigate the safety of a double Lyo-secretome intra-articular

injection, and it will be presented in detail in Part II of the present thesis [46]. The resume of cell-free studies is shown in **Table 4**.

Author	# Patients	Group/treatment	Joint	Donor/source	Injection route	Dose	# Injections	Evaluations	Side effects	Outcome	Follow-up
Hunakova et al., [51]	6 (12 joints)	allo Ad-MSCs CM: 6 (12 joints)	elbow	Allogeneic Ad-MSCs conditioned medium	IA	not specified	2	Synovial fluid and ROM	NO	positive	42 days
Mocchi et al., [46]	3 (6 joints)	Lyosecretome in HA: 3 joints Mannitol in HA: 3 joints	elbow, knee	Allogeneic Ad-MSCs conditioned medium	IA	2x10 ⁶ cell equivalents	2	Clinical and orthopedic parameters, owner questionnaire	NO	Safety evaluation	180 days

Table 4. Presentation of studies on naturally occurring OA applying MSCs-derived cell-free therapies in dogs.

In most naturally-occurring OA studies, the safety of MSCs-based therapy was assessed for both endovenous and intra-articular injections without reporting adverse effects in the majority of the papers [46–54,58–61,63–69,71–76,78]. Few mild side effects, such as self-limiting joint inflammation with lameness worsening, were observed by the minority of the studies [55–58,62,70]. Regarding the efficacy, pre- and post-clinical evaluations usually included orthopaedic examinations assessing lameness, pain, range of motion and other functional parameters evaluated by the veterinarian using numeric validated scales [46–55,57,58,60–62,64–67,71–75], while objective gait analysis with force or pressure platforms, accelerometry or clinical metrology instruments was assessed only in a few papers [47–49,59,61–63,68–70,75]. Furthermore, diagnostic imaging [47,48,53,60,66,67,71–74,76,78] and synovial fluid analyses [48,59,63,72,73,76,51] can be added for more information. Additionally, the efficacy evaluation may include validated owner questionnaires, such as Canine Brief Pain Inventory (CBPI) or the Helsinki Chronic Pain Index (HCPI) [46–50,52–56,58,59,61,62,64–66,69,71,73,75]. The owner's point of view is indeed a sensitive instrument to quantify treatment response because the dog's daily behaviour in its home environment appears to be superior to the periodic assessment in clinical settings [58]. However, changes in the home environment and owner bias may influence the outcome measures.

MSCs-based therapy for equine naturally occurring osteoarthritis

Several experimental studies have been conducted regarding regenerative medicine therapies applied to musculoskeletal diseases (MSDs) in horses, including the treatment of osteoarthritis with PRP, autologous serum and MSCs-based products [79–85]. Overall, the literature supported the high effectiveness of these products in osteoarticular injuries in equine models, due to their regenerative and anti-inflammatory properties, making them a promising therapeutic alternative [86].

From a systematic literature review, only five main studies treated naturally occurring equine OA with MSCs [87]. Different breeds, sexes, ages, and levels of performance were included in the studies. Moreover, OA occurred in different joints, ranging from low to high-motion joints [88–91]. The AAEP (American Association of Equine Practitioners) score was used in the majority of the papers for the lameness evaluation [88,90,91], while own clinical score was used in the remaining works [89,92]. The other clinical parameters evaluated to assess safety and efficacy were the flexion test, joint effusion, heat at palpation and pain to pressure [89–92]. In most studies, an owner survey was used to check the health status and degree of lameness and the general horse improvement in sportive performance [88,89,91,92]. All the studies cited below used the intra-articular injection as a route of MSCs administration. Among them, Ferri et al. assessed the postoperatively IA injection of autologous bone marrow-derived MSCs resuspended in hyaluronic acid in 33 patients with stifle OA, showing promising results over a 24-month follow-up [88]. Also, allogeneic umbilical cord-derived MSCs have been investigated in a prospective blinded placebo-controlled study to treat metacarpal- or metatarsophalangeal joint, with positive outcomes for over 6 months [92]. Blindness in a clinical trial is associated with a low bias rate. However, the blinded control period can be maintained only for a short period to ensure the study design, as it is very difficult to obtain longer permission from horse owners for this type of study [87].

The remaining three articles have been published by Broeckx and colleagues, starting from a pilot study to a preliminary study, to a final randomized, multicenter, double-blinded, and placebo-controlled study. They demonstrated positive results associated with the use of allogeneic peripheral blood-derived MSCs chondrogenically induced compared to the "native" MSCs, combined with PRP in the first phases of the study, then resuspended in allogeneic equine plasma as the final investigational veterinary product (IVP) [89–91]. Equine naturally occurring OA studies are resumed in **Table 5**.

Reference	# Patients	Group/treatment	Joint	Donor/source	Injection route	Dose	# Injections	Evaluations	Side effects	Outcome	Follow-up
Ferris et al., [88]	33	MSCs in HA: 33	Stifle	Autologous BM-MSCs	IA	15-20x10 ⁶	1	owner evaluation (if not possible, veterinarian or trainer evaluation)	YES (3 horses transient joint flare)	partially positive	24 months
Broeckx et al., [89]	165	MSCs+PRP: 49 iMSCs+PRP: 116	different joints	Allo Blood-derived MSCs; chondrogenic induced allo Blood-derived MSCs	IA	IVP	1	clinical evaluations, return to work	YES (3 horses moderate joint flare)	partially positive	18 weeks
Broeckx et al., [90]	20	PRP: 5 MSCs: 5 MSCs+PRP: 5 iMSCs+PRP: 5	fetlock	Allo Blood-derived MSCs; chondrogenic induced allo Blood-derived MSCs	IA	IVP	1	clinical and orthopedic evaluations	NO	positive	12 months
Marinas-Pardo et al., [82]		MSCs: 80	different joints	Allogeneic Ad-MSCs	IA	IVP	1 to 3	clinical and orthopedic evaluations	NO	positive	90 days
Broeckx et al., [91]	75	MSCs:75	fetlock	allo blood-derived iMSCs+equine allogeneic plasma	IA	IVP	1	clinical and orthopedic evaluations, owner evaluations	NO	positive	12 months
Magri et al., [92]	28	MSCs: 28	Metacarpo/meta tarso-phalangeal joint	Allogenic umbilical cord-derived MSCs	IA	10x10 ⁶	2	clinical, orthopedic, radiographic and owner evaluations	YES (mild joint inflammation in 4 horses)	positive	6 months

Table 5. Presentation of studies on naturally occurring OA applying MSCs in horses.

Regarding the side effects, three studies observed a mild to moderate inflammatory response after intra-articular injection with MSCs in a few patients [88,89,92]. Overall, the cited studies showed a positive and constant improvement for at least several months. However, more randomized, controlled, blinded and long-term studies are needed to make further conclusions. It is crucial to determine the exact composition and effect of all orthobiologic therapeutics to develop effective and standardized treatment protocols [87]. The emerging field of cell-free therapies represents a promising approach to treating joint disorders in horses. In 2022, in an in vitro experimental study, EVs derived from bone marrow-, adipose tissue-, and synovial fluid-derived MSCs were tested on inflamed equine chondrocytes to evaluate their immunomodulatory activities, showing abilities to reduce the expression of metalloproteinases [83]. The same year, an in vivo experimental study assessed the anti-inflammatory effects of IA injection of allogeneic secretome derived from BM-MSCs in eight equine models of induced joint inflammation. The findings were compared with MSCs treatment: no significant differences were observed between the two groups, even though a positive tendency was observed in the secretome group regarding joint perimeter and synovial fluid analysis [84]. These results suggested that MSCs derived secretome and EVs could reduce joint inflammation. However, at the time of writing, no in vivo studies on naturally occurring OA have been conducted in horses.

Objectives

The first aim was the validation of the technological process for veterinary clinical-use medicinal production on equine and canine batches of Lyo-secretome, prepared starting from adipose tissue-derived MSCs, collected from healthy donors meeting all the requirements for clinical use. Secretome production was induced by 24h of serum starvation: cells were then detached and counted to calculate cell equivalents, and the supernatants were concentrated and filtrated before the final freeze-drying step. Microbiological tests were performed during all the production phases, as well as sterility tests for injectable pharmaceutical products. The quantitative and qualitative analysis included the assessment of physiochemical properties, extra-cellular vesicle morphology, size distribution, surface biomarkers, and protein and lipid content. Furthermore, in vitro metabolic tests were performed confirming no cytotoxic effects, while positive proliferative effects on target cells, such as tenocytes, chondrocytes, and synovial fluid derived-MSCs were demonstrated. Additionally, proteomic analysis demonstrated the presence of proteins involved in regulating the inflammatory response.

Based on these results, the next step was the in vivo safety and efficacy evaluation of double intra-articular injection of Lyo-secretome resuspended in hyaluronic acid. A double-blinded randomized controlled clinical trial was performed enrolling twenty-two canine patients spontaneously suffering from naturally occurring elbow or knee osteoarthritis. Clinical evaluations and owner questionnaires have been used to monitor the patients during the follow-up. For the equine species, a preliminary in vivo safety evaluation was performed on five horses suffering from spontaneous fetlock osteoarthritis, treated with crescent doses of Lyo-secretome resuspended in hyaluronic acid.

Part I

Objective - Equine Lyo-secretome production, characterization, and proteomic analysis

Resume – Validation of the technological process for veterinary clinical-use medicinal production on three different batches of Lyo-secretome, prepared starting from adipose tissue collected from three healthy donors meeting all the requirements for clinical use. Secretome production was induced by 24h of serum starvation: cells were then detached and counted to calculate cell equivalents, and the supernatants were concentrated and filtrated before the final freeze-drying step. Microbiological tests were performed during all the production phases. The quantitative and qualitative analysis included the assessment of physiochemical properties, extra-cellular vesicle morphology, size distribution, surface biomarkers, and protein and lipid content. Furthermore, in vitro metabolic tests were performed confirming positive effects on target cells, and a proteomic analysis demonstrated the presence of proteins involved in the regulation of the inflammatory response.

Published article - **Equine Mesenchymal Stem/Stromal Cells Freeze-Dried Secretome (Lyosecretome) for the Treatment of Musculoskeletal Diseases: Production Process Validation and Batch Release Test for Clinical Use.** Michela Mocchi, Stefano Grolli, Silvia Dotti, Dario Di Silvestre, Riccardo Villa, Priscilla Berni, Virna Conti, Giulia Passignani, Francesca Brambilla, Maurizio Del Bue, Laura Catenacci, Milena Sorrenti, Lorena Segale, Elia Bari, Pierluigi Mauri, Maria Luisa Torre, and Sara Perteghella

Key aspects - An intra-articular injectable pharmaceutical powder has been produced, the technological process has proven to be consistent, and a panel of quality tests has been defined. Lyosecretome demonstrated a direct activity on different musculoskeletal cell types such as tenocytes, chondrocytes, and tissue-resident MSCs; also, the proteomic composition suggested a possible role of Lyosecretome for regenerative medicine applications.



Article

Equine Mesenchymal Stem/Stromal Cells Freeze-Dried Secretome (Lyosecretome) for the Treatment of Musculoskeletal Diseases: Production Process Validation and Batch Release Test for Clinical Use

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Abstract: In the last decades, it has been demonstrated that the regenerative therapeutic efficacy of mesenchymal stromal cells is primarily due to the secretion of soluble factors and extracellular vesicles, collectively known as secretome. In this context, our work described the preparation and characterization of a freeze-dried secretome (Lyosecretome) from adipose tissue-derived mesenchymal stromal cells for the therapy of equine musculoskeletal disorder. An intraarticular injectable pharmaceutical powder has been formulated, and the technological process has been validated in an authorized facility for veterinary clinical-use medicinal production. Critical parameters for quality control and batch release have been identified regarding (i) physicochemical properties; (ii) extracellular vesicle morphology, size distribution, and surface biomarker; (iii) protein and lipid content; (iv) requirements for injectable pharmaceutical dosage forms such as sterility, bacterial endotoxin, and Mycoplasma; and (v) in vitro potency tests, as anti-elastase activity and proliferative activity on musculoskeletal cell lines (tenocytes and chondrocytes) and mesenchymal stromal cells. Finally, proteins putatively responsible for the biological effects have been identified by Lyosecretome proteomic investigation: IL10RA, MXRA5, RARRES2, and ANXA1 modulate the inflammatory process RARRES2, NOD1, SERPINE1, and SERPINB9 with antibacterial activity. The work provides a proof-of-concept for the manufacturing of clinical-grade equine freeze-dried secretome, and prototypes are now available for safety and efficacy clinical trials in the treatment of equine musculoskeletal diseases

Keywords: mesenchymal stem cells; secretome; regenerative medicine; musculoskeletal disorders; equine

1. Introduction

Musculoskeletal disorders (MSDs) have an extensive and growing impact representing a major health problem worldwide. Despite years of research, the understanding of these diseases and the control on their progression has not yet been achieved; indeed, different therapeutic approaches are being investigated to prevent the disorders or to promote recovery and/or regeneration of the musculoskeletal system, including muscles, bones, and joints [1,2]. MSDs often result from traumatic injuries due, for example, to vigorous physical activity leading to excessive muscle fatigue, damage of joint structures (articular cartilage, subchondral bone, synovial membrane), and inflammation or lesion of the tendon/ligament structure. Structural and genetic factors are also associated with musculoskeletal diseases onset and progression [3]. MSDs represent a high percentage of orthopedic clinical pathologies in veterinary practice and, mainly, in equine practice. Due to the low healing capacity of tissues involved in joint and tendon disorders, there is a tendency to develop chronic diseases with a significant clinical relevance [4]. Beyond the traditional treatments (local or systemic nonsteroidal anti-inflammatory drugs, intralesional steroids, correct shoeing, chondroprotectants such as hyaluronic acid), innovative regenerative therapies, including the use of Mesenchymal Stem/Stromal Cells (MSCs), have emerged over the past ten years as promising agents to promote local repair. Especially in the veterinary field, many clinical applications of cell-based therapies have been described, starting from the early 2000s with the first application of MSCs on the equine model [5,6]. Despite the considerable amount of data suggesting the safety and efficacy of MSCs in experimental animal models and preclinical studies, cell-based therapies are not yet routinely applied in the clinic [7–10]. Over the years, the therapeutic application of MSCs has known a paradigm shift: Nowadays, their engraftment, proliferation, and differentiation properties are not considered key features of their therapeutic abilities. Instead, the main actors of MSCs benefit are now considered several bioactive molecules (including proteins, cytokines, chemokines, growth factors, but also nucleic acid) released by cells and involved in cell-to-cell communication and cross-talk [11]. Known as secretome, this complex set of secreted factors and vesicles seems to represent a valid alternative as a cell-free therapy, offering several advantages in comparison to cell-based therapy, such as lower immunogenicity, ease of storage and handling, and lower cost ensuring usefulness and feasibility in the clinic [12,13]. However, addressing a suitable medicinal product is challenging, and further investigations are needed to shed light on secretome composition, properties, and in vivo behavior. Indeed, while evaluating secretome safety and efficacy for clinical use, many aspects, ranging from production processes to molecular characterization, must be defined.

In this study, an injectable freeze-dried pharmaceutical powder containing equine secretome has been formulated (Lyosecretome); the technological process has been validated in an authorized facility for veterinary clinical-grade medicinal production. Critical parameters for quality control and batch release have been identified regarding (i) physicochemical powder properties; (ii) extracellular vesicle morphology, size distribution, and surface biomarkers; (iii) protein and lipid content; (iv) requirements for injectable pharmaceutical dosage forms (sterility, bacterial endotoxin, and Mycoplasma); and (v) in vitro potency tests, and efficacy on proliferative induction of selected cell lines. Finally, putative proteins responsible for the biological effects have been identified by a Lyosecretome proteomic investigation based on nano Liquid Chromatography coupled with high-resolution mass spectrometry.

2. Results

2.1. Lyosecretome Production

Three different equine AD-MSCs lines from the biobank meet all the requirements for clinical use as all the steps were made according to ISO 9001:2018 clinical grade. Secretome production was induced by 24 h serum starvation; after this time, the supernatants were collected, and cells were detached to estimate their total amount and viability (Table 1).

Table 1. Lyosecretome batch production from three different equine AD-MSCs lines.

Batch n.	Total Cell Number $\times 10^6$	Cell Viability (%)
1	224	99
2	170	98
3	65	99

The supernatants were first centrifuged to eliminate cell debris, followed by an ultrafiltration process to concentrate and filter. The concentration step allows to reduce the volume, reaching a fixed value of cell equivalents per mL (0.5×10^6 cell/mL); instead, the filtration phase with 5 kDa molecular weight cut-off allows one to retain both EVs and soluble proteins contained in the supernatants. At the end of the concentration step, the obtained volume was diafiltered at least five times with ultrapure deionized water to erase any tracks of undesired compounds. The starting volume for all the produced batches was about 800 mL, the time process lasted approximately 3 h using a filtration module with a superficial area of 370 cm², and the calculated ultrafiltration efficiency was 17.8 ± 2.3 L/m²/h (mean value $\pm$ standard deviation, $n = 3$). Once the purified solution was obtained, mannitol was added as the selected cryoprotectant. Samples were stored at -80 °C before the freeze-drying process. In this timeframe, to ensure the quality of the product, microbiological tests were performed during all the production phases to prove that the sterility conditions were maintained during the formulation along with the final product. Microbiological tests were conducted by an accredited and certified facility (IZSLER, Brescia, Italy), which routinely controls cell and cell-derived products for veterinary clinical use. These tests affirmed: (i) The absence of any mycoplasma and (ii) bacterial endotoxin level in an acceptable range (Table 2).

Table 2. Lyosecretome microbiological test: Sterility, bacterial endotoxin, and Mycoplasma.

Batch n.	Sterility	Bacterial Endotoxin	Mycoplasma
1	compliant	3.4 Eu/mL	no presence
2	compliant	3.3 Eu/mL	no presence
3	compliant	3.4 Eu/mL	no presence

2.2. Lyosecretome Characterization

After the lyophilization process, a technological characterization was performed on Lyosecretome powder.

To check the suitability of each formulation for a future in vivo administration, further analyses were performed on the final product. For each formulation, cake's appearance was evaluated by randomly selecting four vials and performing a visual inspection. Following the aqueous solvent's evaporation during lyophilization, a solid and porous cake was formed, free of defects. The lyophilized secretome dissolved rapidly by a gentle agitation adding water, generating a clear suspension free of visible particles. The osmolarity was always within the acceptable range for injectable formulations (250–350 mOsm/Kg) and always between 320 and 350 mOsm/Kg. The pH value was always in the range of 7.2 to 7.6.

FTIR spectra were performed on Lyosecretome batches. Comparing the three batches reported in Figure 1, the spectral region goes from 650 to 4000 cm⁻¹; the batches, as expected, have similar bands at around at 1082 cm⁻¹ and 1019 cm⁻¹ and 930 cm⁻¹; those picks are predominant, and unfortunately, in this region, there is an overlap with the characteristic mannitol vibrations bands; thus, it is difficult to distinguish the Lyosecretome presence (Figure 1). Lyosecretome bands were also detected in the spectral region between 1423 cm⁻¹ and 1458 cm⁻¹, pointing out the presence of CH₂ groups and 1374 cm⁻¹ and 1375 cm⁻¹ related to CH₃ groups, thus confirming the presence of lipids and protein.

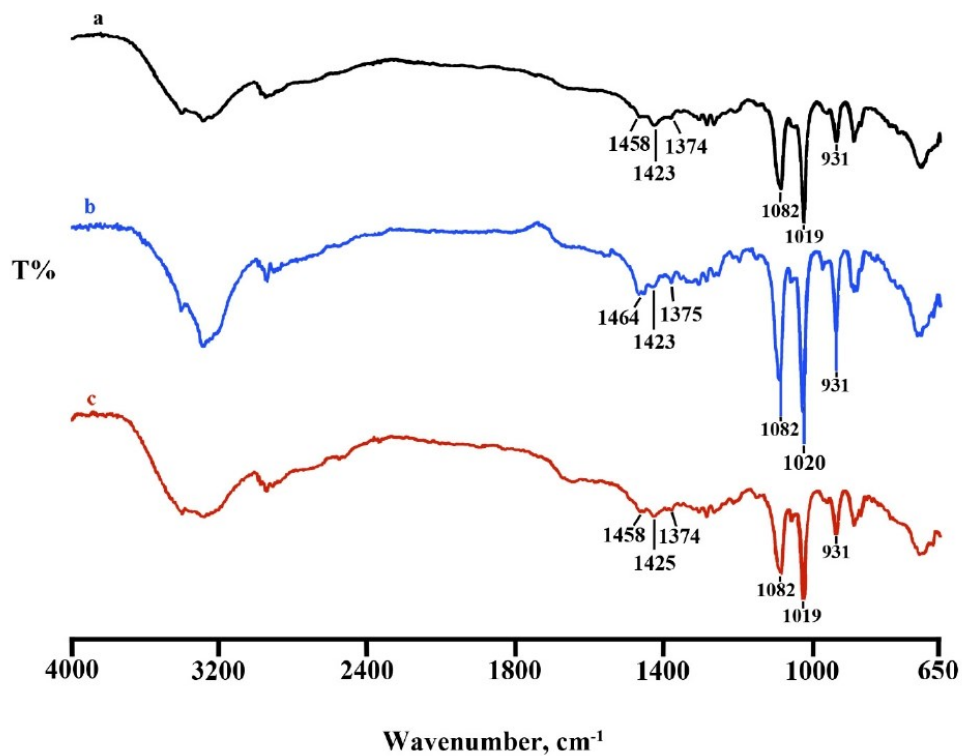


Figure 1. Comparison between FTIR spectra of Lyosecretome batch 1 (spectrum a), 2 (spectrum b), and 3 (spectrum c), respectively.

The three batches were analyzed by DSC, and the thermal profile was reported below as a comparison (Figure 2). The endothermic peak at 164.3 ± 0.8 °C ($\Delta H = 278 \pm 3$ Jg^{−1}) was due to the melting of crystalline mannitol (β form), followed by sample decomposition.

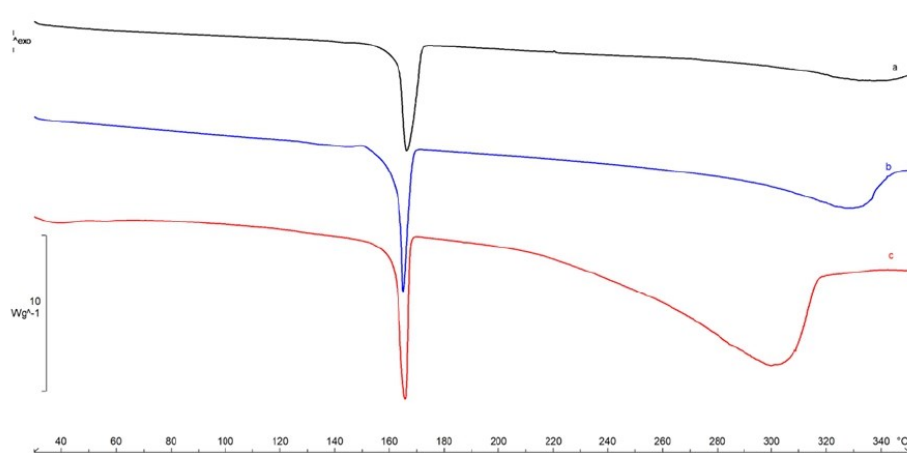


Figure 2. DSC profiles of Lyosecretome batches 1, 2, and 3 (curves a, b, and c).

The anhydrous nature of the lyophilized samples was confirmed by TGA analysis (Figure 3), where no mass losses were recorded in the temperature range 50–100 °C, related to residual water. An almost complete mass loss was recorded for all the samples starting from about 244 °C due to sample decomposition, confirming the same thermal stability for the three batches.

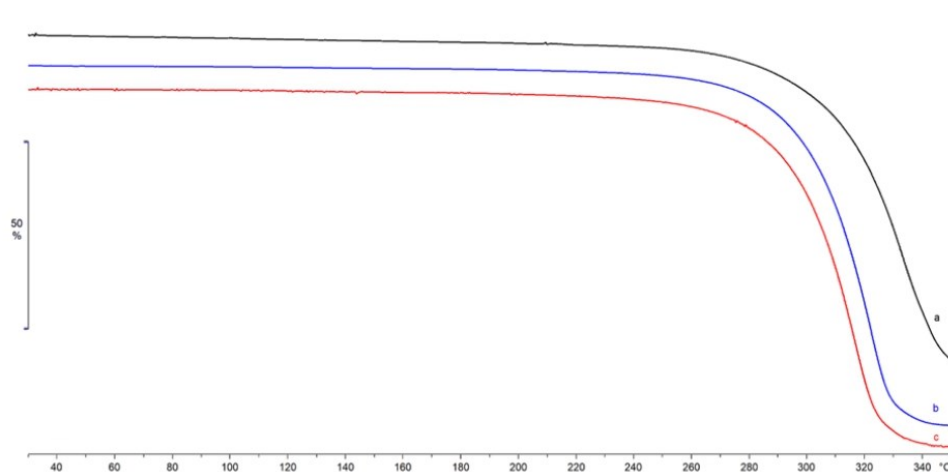


Figure 3. TGA profiles of Lyosecretome batches 1, 2, and 3 (curves a, b, and c).

Morphological investigation through SEM images showed that vesicles integrity and spherical shape were maintained for all three batches, demonstrating that ultrafiltration and lyophilization processes did not influence particle structure (data not presented).

Particle size characterization has been performed on Lyosecretome using NTA technology to (i) count nanoparticles observed in a specific volume, (ii) measure the diameter, and (iii) obtain the particle concentration. The analysis detected a heterogeneous population including nano/micro-structured vesicles; in detail, for all three batches, 10% of the population diameter (d_{10}) ranged 77–95 nm, 50% of the population diameter (d_{50}) ranged 115–156 nm, and 90% of the population diameter (d_{90}) was less than 260 nm. Regarding particle concentration, it ranged between 1.77×10^8 and 3.47×10^8 particle/mL based on the considered batches (Table 3 and Figure 4).

Table 3. Particle size distribution and concentration of batch 1 and 2 (mean values $\pm$ standard deviation, $n = 3$).

Batch n.	Mean (nm)	Mode (nm)	d_{10} (nm)	d_{50} (nm)	d_{90} (nm)	Concentration (Particle/mL)
1	142.7 ± 4.9	81.0 ± 2.8	77.5 ± 2.1	117.5 ± 3.2	231.2 ± 9.5	$2.46 \times 10^8 \pm 1.48 \times 10^7$
2	198.5 ± 6.4	114.8 ± 11.8	94.8 ± 1.6	156.7 ± 8.6	253.5 ± 18.5	$3.47 \times 10^8 \pm 2.66 \times 10^7$
3	187.1 ± 3.9	94.9 ± 3.0	91.5 ± 2.2	151.2 ± 2.8	261.3 ± 7.4	$1.77 \times 10^8 \pm 7.77 \times 10^6$

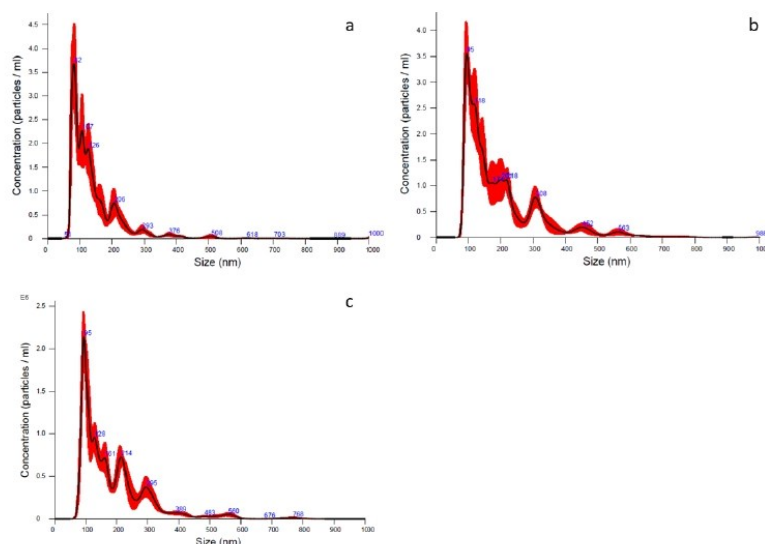


Figure 4. NTA analyses showing averaged particles concentration and size distribution of batch 1 (a), 2 (b), and 3 (c), respectively. The average of each batch was made by processing 6 cycles of 60 s each. The red part indicates the $\pm$ standard error of the mean values ($n = 3$). Particles concentration is expressed as mean value $\pm$ standard deviation ($n = 3$).

The number and size of exosomes were determined by ExoView with biomarker detection. This method is based on a direct-biomarker association that captures particles into a chip. ExoView also allows discriminating with fluorescence if a protein is expressed on the surface or into the exosome. The analysis method could be divided into two macro steps: Interferometric imaging that gives information regarding the exosome's number and size, and fluorescence analysis that gives the intensity of a positive-marker protein. The data accuracy depends on correct binding between proteins expressed by exosomes and extracellular vesicles and their specific antibodies marker. As a result, a first consideration was a correlation between the horse's protein and human protein: Data confirm that human capture antibodies had a fixed animal ligand. Moreover, the average colocalization counts are shown in Figure 5 (triplicate analysis). The correlations between proteins expressed on exosome's surfaces and fluorescent antibody markers can be vertically read in the chart. The last plot column indicates the negative control performed by Murine IgG. In the first plot, column data show that in a horse's secretome, there is a high number of exosome's surface proteins responsive to the anti-CD-9 fluorescent human antibody, and the second most-expressed ligand is a protein that could be correlated to anti-CD-81 fluorescent human antibody. Finally, the lower capture marker is the anti-CD-63 fluorescent human antibody. There is also double or triple positive binding between the surface protein and human antibodies, as the seven exosomes are simultaneously expressed on the external membrane proteins positive to anti-CD-81 and anti-CD-63 fluorescent human antibodies.

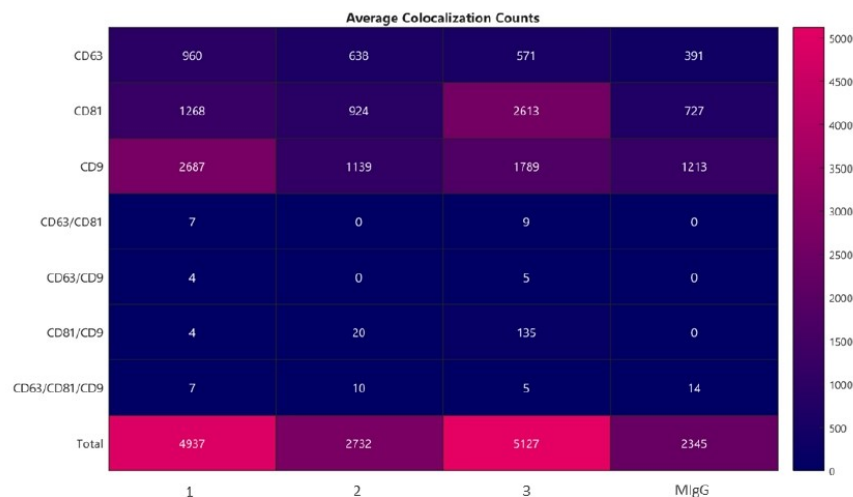


Figure 5. Average colocalization counts evaluating the correlations between proteins expressed on exosome's surfaces and fluorescent antibodies markers CD63, CD81, and CD9. According to the average results of the three determinations (without considering the negative control murine IgG), it is possible to underline that there were 723 exosomes positive only for the anti-CD-63 human antibody, approximately 1601 exosomes positive only for the anti-CD-81 human antibody, 1871 exosomes positive only for the anti-CD-9 human antibody, 5 exosomes have a double positive match for anti-CD-81 and anti-CD-63 human antibodies, approximately 5 exosomes have a double positive match for anti-CD-63 and anti-CD-9 human antibodies, 53 exosomes are simultaneously positive for anti-CD-9 and anti-CD-81 human antibodies, and 7 exosomes present a triple positive match for human antibodies.

Lyosecretome was characterized in terms of total lipid and protein content, and results are reported in Table 4 expressed as μg of protein or lipid per mg of Lyosecretome.

Table 4. Lyosecretome total protein and lipid content; mean values $\pm$ standard deviation, $n = 3$. Different letters indicate a significant difference between groups ($p < 0.0001$).

Batch n.	$\mu\text{g Proteins/mg Lyosecretome}$	$\mu\text{g Lipids/mg Lyosecretome}$
1	10.0 ± 0.07 a	0.7 ± 0.05 a
2	13.0 ± 0.20 b	1.0 ± 0.04 b
3	10.6 ± 0.02 c	0.6 ± 0.07 c

Focusing on the yield of each batch, for batch 2, we obtained a higher amount of both protein and lipid content, and comparing the three batches overall, a significant difference was found ($p < 0.05$). Moreover, it is evident that there was a correlation between protein and lipid content. It is well known that cell lines can highly affect secretome production and composition due to the variability within the same species. This aspect could explain the results in terms of different yield, although a certain reproducibility is maintained.

It should be noticed that the methods applied to analyze protein and lipid content meet the pharmaceutical conditions and were previously validated [14]. For BCA-protein quantification, the mean coefficient of calibration curve determination was in a range of 0 up to $100 \mu\text{g/mL}$ of BSA in water; the curve equation has an intercept not statically significant, and the plot of the residuals had a common distribution of the error. These latter considerations mean that no systematic error influenced the calibration (data not shown). Considering the lipid quantification that was performed by a Nile Red assay, the mean coefficient of the calibration curve determination was within a range of $0\text{--}20 \mu\text{g/mL}$ of PC in water; the distribution of the plot residuals had a normal trend of the error; thus, no systematic error influenced the calibration (data not shown).

2.3. In Vitro Potency Test

The anti-elastase activity was tested on equine Lyosecretome at different concentrations (2, 5, 10, and 20 mg/mL), showing a dose-dependent trend (Figure 6). The activity assay consists of a kinetics enzymatic reaction considered from time 0 up to 40 min. The activity exhibited almost 50% at the higher concentration, considering Epigallocatechin gallate as a positive control due to its positive inhibition of the elastase enzyme.

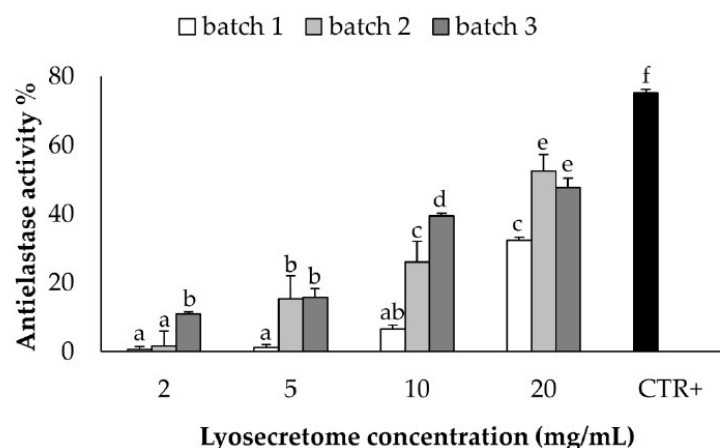


Figure 6. Anti-elastase activity was tested for the three batches at different Lyosecretome concentrations: 2, 5, 10, and 20 mg/mL, obtaining a dose-dependent trend. Mean values $\pm$ LSD ($n = 3$), ANOVA. Different letters (a, b, c, d, e, and f) indicate significant differences between the means ($p < 0.05$), whereas the same letter indicates no significant difference ($p > 0.05$).

The metabolic activity of equine AD-MSCs, SF-MSCs, tenocytes, and chondrocytes was evaluated after treatment with Lyosecretome. In detail, Lyosecretome was tested at the concentrations of 400,000, 200,000, and 100,000 cell equivalents/well. For all cell types, the exposition to Lyosecretome stimulated cell proliferation compared to serum-free medium ($p < 0.0001$), and a dose-dependent trend of cell metabolic activity was observed. The effects of Lyosecretome treatment were statistically different in the four cell types ($p < 0.0001$). As shown in the figure below (Figure 7), the dose-dependency reached a plateau at 200,000 cell equivalent/well. The medium with 10% FBS added was considered a positive control, while the serum-free medium was used as a negative control.

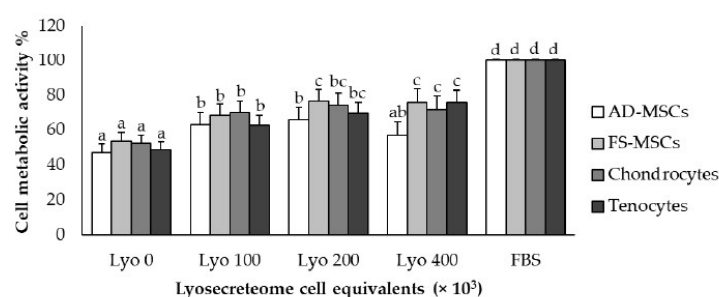


Figure 7. Cell metabolic activity on AD-MSCs, SF-MSCs, tenocytes, and chondrocytes tested at different Lyosecretome concentrations demonstrating a dose-dependency up to 200,000 cell equivalents/well. Mean values $\pm$ LSD ($n = 4$), ANOVA. Different letters (a, b, c, d) indicate significant differences between the means ($p < 0.05$), whereas the same letter indicates no significant difference ($p > 0.05$). FBS indicates the positive control, while Lyo 0 stands for the negative control.

2.4. Proteomic Investigation

The protein characterization of the secretome (Lyosecretome) isolated from equine mesenchymal stem cells allowed the identification of 647 distinct proteins (Table S1). This proteome was defined by seven technical replicate analyses performed with two different mass spectrometers. Regardless of the instrumentation used, all replicates showed good repeatability with R^2 values ranging from 0.92 to 0.99 (Figure 8A). About 25% of the proteins were identified by an average spectral count > 1 (Figure 8B). In addition, 95 proteins were identified in all analyzed samples, while 272 were found in at least 2 replicate analyses out of 7 (Table S1).

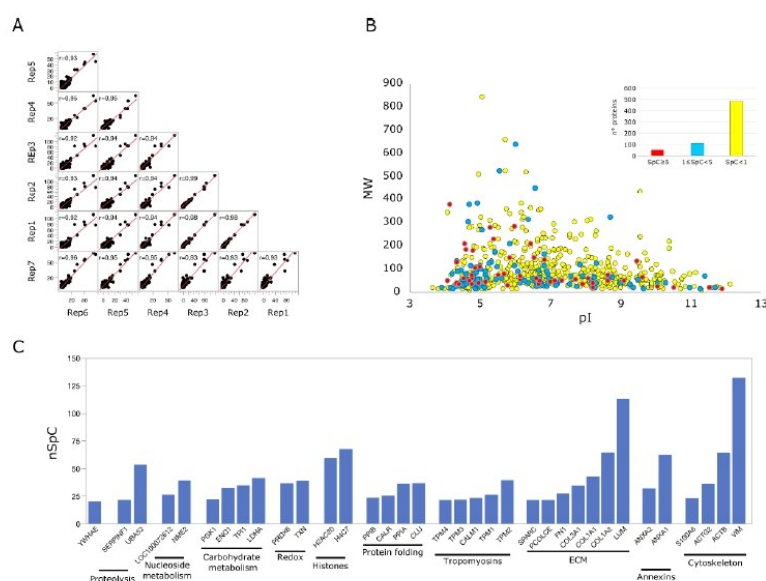


Figure 8. Proteomic analysis secreted from equine mesenchymal stem cells. (A) Spearman's correlation computed using Spectral counts of replicate LC-MS/MS analysis. (B) Virtual 2D-Map (pI vs. MW) of the total proteins identified ($n = 647$); color codes indicated the average spectral counts characterizing the identified proteins. (C) Rank list of the most abundant identified proteins ($n = 35$, $nSpC > 20$) clustered for functional categories.

The rank list of the most abundant identified proteins (Figure 8C) highlighted the presence of well-defined functional categories. Those most represented included ECM, Tropomyosins, Cytoskeleton, Carbohydrate metabolism, and Proteins folding. However, although less represented, other interesting proteins involved in Proteolysis regulation, Redox homeostasis, Histone proteins, and Annexins were found. All these categories agreed with those (Biological Process, Molecular Function, and Cellular Component) most enriched by considering the global characterizes protein profile (Figure S1); interestingly, a relevant enrichment of proteins involved in chaperone-mediated protein folding emerged from this analysis. A major level of detail concerning functional categories and proteins most relevant in the Lyosecretome from equine MSCs was reached by reconstructing and processing, at a functional and topological level, an *Equus caballus* protein–protein interaction (PPI) network; it was reconstructed exploiting the high sequence homology (>87%) between *Equus caballus* and *Homo sapiens* protein sequences (Figure S2).

The *Equus caballus* PPI network reconstructed starting from proteins secreted by mesenchymal stem cells counted 269 nodes and 2016 edges. They were clustered in 29 functional/topological modules (Figure 9) further classified in 6 macro-areas, including Structural proteins, Genetic Information Processing, Metabolism, Transport, Proteolysis, and

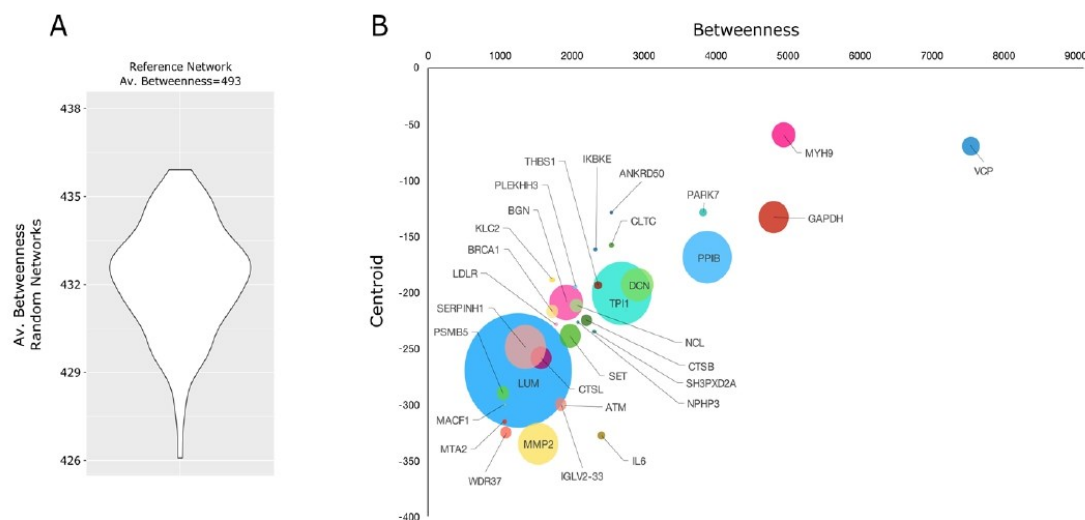


Figure 10. PPI network hubs in PPI network reconstructed from proteins secreted by *Equus caballus* mesenchymal stem/stromal. **(A)** Violin plot reporting the average Betweenness in Random networks. **(B)** PPI network hubs were selected considering Betweenness, Centroid, and Bridging (proportional to circle size) centralities; proteins were defined as hubs if all centrality values were above the average values calculated on the whole network.

3. Discussion

This study aimed to propose a procedure for a scalable production of equine Lyosecretome, define its characteristics, and pave the way for its use in in vivo preclinical studies in animals affected by musculoskeletal disorders. To this aim, secretome production was induced by serum starvation of expanded AD-MSCs, and then the freeze-dried secretome was characterized in terms of qualitative and quantitative parameters. Experimental evidence indicates that MSCs can be isolated from almost every tissue of the body [15], even if it is still unclear whether cells derived from different sources share the same biological and therapeutic features. However, compared to the Lyosecretome prepared from MSCs isolated from human adipose tissue, we found similar categories, including genetic information processing, immune response, and stress response. Adipose tissue and bone marrow are the preferred sources to isolate and expand MSCs for equine clinical applications [13]. In our study, AD-MSCs were used for the preparation of Lyosecretome.

Ultrafiltration was chosen for the easy scalability to prepare large batches because it presents several advantages compared to other techniques commonly used to isolate MSC-secretome, including the affordable costs and the shorter time requested by the entire process [16]. Furthermore, this technique grants the choice between filtration modules with different molecular weight cut-offs (MWCO), allowing the preferred retention based on the desired yield in small vesicles (as, for example, extracellular vesicles) or the whole secretome. Other conventional methods commonly used for isolating EVs, such as ultracentrifugation, present several limitations regarding the possible damage of EVs membranes, due to the high g-force required by the centrifugation steps [17]. In the current study, we selected a relatively low MWCO able to retain the whole secretome to ensure a large-spectrum therapeutic potential to the preparation, even though several studies support the hypothesis that the EVs fraction alone might be sufficient to promote healing of the injured tissues [18]. A biotechnological product such as secretome can be thermolabile, and thus it cannot be dried applying thermal methods, responsible for possible modification and/or degradation of bioactive components (e.g., protein). For this reason, to reach a water-free product, the lyophilization process was considered. Lyophilization guarantees

the maintenance of sterile conditions when changing from liquid to a powder state; moreover, the final product is easy to store and requires a short reconstitution time of the dried product; long-term stability is also provided [19]. Unfortunately, this technique has several limitations: Costs, limited capacity, long processing time (it lasts approximately 4 days), and difficulties in the “scale up” from laboratory to industrial production. Thus, its use is justified when the product’s value is sufficiently high, such as producing biotechnological drugs, vaccines, vitamins, antibiotics, liposomes, and oncological products. The freeze-drying method creates freezing and drying stresses that can alter the stability of the biological product; hence, before proceeding, the addition of a cryoprotectant is needed. The cryoprotectant guarantees the protection of both protein and vesicles lipidic layer from the damages of ice crystals formed during the freezing step and inhibits vesicle size alteration by aggregation. In this regard, mannitol is the most suitable cryoprotectant due to its important stabilizing effects on freeze-dried pharmaceutical products [19]. Mannitol properties have been widely discussed and investigated [20,21]. Its role is to ensure long-term stability, easy reconstitution, and maintenance of biological features and activities. However, the use of mannitol still remain controversial and many other excipients, such as trehalose, can be adopted instead [22]. In the current work, mannitol was able to avoid vesicles/particles aggregation as revealed by NTA analysis; moreover, it prevented vesicle structure modification in terms of morphology as shown by SEM analysis. This latter aspect was also ensured by applying ultrafiltration process for the secretome isolation, rather than ultracentrifugation, which can possibly break-up vesicles membrane due to the high shear forces.

FTIR spectra revealed similar bands for each of the three batches, where the presence of lipids and protein content was confirmed. Overall, all samples’ spectra were overlapping, thus ensuring the reproducibility of the batch production process. DSC has been employed to demonstrate the purity, polymorphic form, melting point, and thermal behavior of EV lipid bilayers, which can be affected in terms of stability under adverse conditions [23]. The lyophilization process was conducted successfully for all batches, as confirmed by TGA analysis, with a very low water residual and good thermal stability.

Regarding the analysis performed by the ExoView, the method is based on a direct-biomarker association that captures particles into a chip. The technique allows discriminating whether a protein is expressed on the surface of the exosome or in the exosome by fluorescence analysis of the captured particles. Since a specific chip for analysis of equine secretome is not available, the microarray specific to human proteins was used. Although not validated for equine species, the chip is based on highly conserved proteins, and a cross-reaction is plausible. We observed differences between the analysis of the three batches. The number of exosomes presenting a double correlation anti-CD-81/CD-9 human antibody was higher than the other correlations; moreover, CD-9 binding is probably the most affine to horse secretome’s surface protein due to a higher protein match.

The anti-elastase activity tested on equine Lyosecretome provides the rationale for using secretome in regenerative medicine [24]. High protease expression is a feature commonly found in several musculoskeletal disorders contributing to their onset and evolution. In this context, protease inhibitors work, contributing to restoring extracellular matrix homeostasis. Our results showed a dose-dependent trend for each batch. Significant variability was observed between the different preparation, probably caused by an intrinsic biological variability between the cell populations used for their preparation.

In vitro metabolic activity assays are the gold standard for studying cell viability and proliferation. In the present work, MTT assay was performed on four different cell types, i.e., AD-MSCs, SF-MSCs, tenocytes, and chondrocytes, considered potential targets for the use of Lyosecretome in musculoskeletal disorders. No cytotoxic effect was observed after Lyosecretome treatment in any of the four different cell lines, independently of the used concentration. Lyosecretome was able to maintain cell replication when compared to serum-free medium. A dose-dependent effect was observed for each cell line, and statistically significant differences were observed as a function of the cell type. Cell metabolic activity

ranged from 30% to 75% compared to medium supplemented with FBS, with the higher stimulus observed at a Lyosecretome concentration of 200 mg/mL. Interestingly, at this concentration, tenocytes, chondrocyte, and SF-MSCs were more responsive than AD-MSCs, reaching a plateau at 200 mg/mL.

The effect observed following Lyosecretome treatment, in particular elastase inhibition, could be attributable, at least partially, to its protein content. In fact, in addition to proteins involved in stress response, including the Heat Shock Proteins, we found protease/peptidase inhibitors among the protein classes better represented. In particular, the putative relevant role of some of them, such as SERPINs, was also suggested by their presence among the most abundant proteins (SERPIN1F) and proteins defined as hubs (SERPINH1). Moreover, about 10% of the identified proteins were annotated to be involved in the inflammatory response. In addition to SERPINE1, annotated to also be involved in “Response to bacterium”, the presence of proteins with anti-inflammatory activity (IL10RA, LTA4H, MXRA5 [25], RARRES2 [26,27], TEK, ANXA1 [28]) emerged. Some of them, including MXRA5 (Chemerin), have been previously associated with osteoarthritis, and anti-inflammatory and anti-fibrotic properties have been proposed for this protein suggesting its potential role in chronic inflammation [29]. In this scenario, MACF1 has also been detected in Lyosecretome; this protein plays a core function in wound healing cell migration [30,31], primarily due to its ability to coordinate interaction between actin, microtubule, and cell junctions. Concerning the cytokine profile, several proteins with cytokine/chemokine activity have been observed in the Lyosecretome, i.e., IL-6, IL-7, CSF-1, IL10RA, IL12RB2, CXCL6, GDF7, and Wnt5B. IL-6 and IL-7 are two pro-inflammatory cytokines involved in MSCs immunomodulation [32,33]. The presence of IL-6 agrees with a recent paper by Bundgaard et al. that also demonstrated the presence of CXCL6 in equine bone marrow-derived MSCs [34]. Pro-inflammatory and chondrogenic treatments modulated the expression of both proteins. IL6, acting as a pro-inflammatory mediator, contributes to host defense during infection and tissue injury, but anti-inflammatory properties have also been suggested depending on the presence of other cytokines [35]. CSF-1 plays a role in inflammatory processes and promotes bone regeneration and mobilization of vascular and osteogenic progenitor cells [36]. CSF-1 presence has also been demonstrated in equine bone-marrow-derived MSCs secretome [34]. GDF7 and WNT5B are two secreted signaling proteins whose role has been suggested in tenogenic (GDF7) and osteogenic and chondrogenic (WNT5B) differentiation pathways of MSCs [37,38]. IL10RA mediates the immunosuppressive signal of interleukin 10, an anti-inflammatory cytokine, already observed in bone marrow-derived human MSCs secretome [39].

Highly expressed proteins belonging to the class I small leucine-rich proteoglycan (SLRP) family, including LUM (lumican), DCN (decorin), and BGN (biglycan), were found among proteins defined as hubs. SLRP family comprises a group of extracellular matrix components distributed in most tissue whose gene expression is modified in OA [40]. Lumican, one of the most abundant proteins found in the secretome, regulates collagen fibril organization, and its expression is reduced in OA cartilage [40]. Interestingly, lumican is a major component of the corneal stroma, and its therapeutic use has been proposed for ocular lesions [41]. Furthermore, lumican is considered a key effector in normal wound repair [42]. Decorin plays a role in fibril assembly in tension-bearing tissues. It may contribute to stabilizing the cartilage matrix in OA [43]. Decorin upregulation has been proposed as an attempt to reduce aggrecan fragmentation in post-traumatic OA, and decorin-based therapeutics have been suggested to attenuate OA progression [44]. Concerning biglycan, this extracellular matrix component contributes to the regulation of chondrogenesis and ECM turnover in OA [45]. Interestingly, the Lyosecretome prepared from equine adipose tissue-derived MSCs shares with the equine bone-marrow-derived MSCs secretome described by Bundgaard et al. [34] a number of proteins related to cartilage biology, i.e., LUM, DCN, COMP (cartilage oligomeric matrix protein), SCRGI (stimulator of chondrogenesis-1), EPYC (Epiphygan), GAS6 (growth arrest specific 6).

Regarding Lyosecretome as a pharmaceutical product, the following acceptance criteria were defined: (i) Fulfilment of specific requirements by the cellular source: Viability over 98%, capable for trilineage differentiation and negative for bacterial/viral contamination. (ii) Protein and lipid content were between 10–13 and 0.6–1 µg per mg of powder, respectively, mean EV size in the range of 100–200 nm, EV concentration in the range of $1\text{--}4 \times 10^8$ particles when re-suspended in dH₂O at 1 mg/mL, expression of CD-9, CD-63, and CD-81 markers, negative for bacterial/viral contamination, water content less than 2%, fulfilment of the criteria required for freeze-dried (cake aspect) and injectable formulations (osmolarity, pH, absence of visible particles after resuspension in physiological solution). The assessment on the final product has to be performed by characterization techniques that can be validated at pharmaceutical grade and have an acceptable cost to be routinely implemented [14,46,47]. (iii) Functional aspects should be included to demonstrate that the final product, after having undergone all the isolation, purification, and formulation processes, maintains biological activity. In detail, anti-elastase activity was higher than 20% at 20 mg/mL in dH₂O. Finally, due to the limited experience in preparing equine Lyosecretome (these are the first scaled-up batches prepared following ISO 9001:2018 for validation) the source of variability cannot be fully investigated yet. With a more consistent number of batches prepared, it will be possible to adopt specific measures to minimize the batch-to-batch variability.

4. Materials and Methods

The Lyosecretome production was made at an accredited facility to produce and control veterinary drugs for clinical use (Istituto Zooprofilattico Sperimentale Lombardia and Emilia-Romagna (IZSLER, Brescia, Italy). The production protocol has been approved by the Italian Ministry of Health (Prot. n. 0000778 del 15/01/2020 7.1.2.0.0.0/17/2019—AGD 809). Culture media, trypsin, and antibiotics used for cell cultures were purchased from Euroclone (Milan, Italy). A commercial platelet lysate kit (PL) was obtained from Carlo Erba Reagents (Milan, Italy). Fetal Bovine Serum (FBS) was purchased by Gibco-ThermoFisher (Milan, Italy). Chemicals such as mannitol, bovine serum albumin, Nile Red, acetone, collagenase, and phosphatidylcholine were obtained from Merck Life Science S.r.l. (Milan, Italy) unless mentioned.

4.1. Lyosecretome Production

4.1.1. Mesenchymal Stem/Stromal Cell Isolation

Allogeneic AD-MSCs from equine species were obtained from the biobank at Istituto Zooprofilattico Sperimentale Lombardia and Emilia-Romagna (IZSLER) (Brescia, Italy). The clinical data related to the cells stored in the biobank as gender, age, the weight of the donor, and cell passage number, were recorded by the IZSLER. In detail, the secretome was produced by three donors. The donors were 10 years old, male, and the breed is unknown. The samples were tested for Equine Herpesvirus, Equine Arterivirus, Flavivirus, and Lentivirus (antibodies screening). All the samples were collected from animals killed at the slaughterhouse following the related Italian Law. Adipose tissue digestion was performed at 37 °C, 5% CO₂ using type II collagenase 0.075% (*w/v*) solubilized in Phosphate Buffer Saline (PBS) with Ca²⁺ and Mg²⁺ supplemented with penicillin (50 U/mL), streptomycin (20 µg/mL), and amphotericin B (2.5 µg/mL). After 1 h, Dulbecco's modified Eagle's Medium (DMEM) with 10% (*v/v*) of equine platelet lysate was added to the cell suspension in a 1:1 ratio. The digested tissue was filtered with 70 µm filters and centrifuged at 600 × *g* for 5 min. The recovered stromal vascular fraction was grown in a monolayer (100,000 cells/cm²) in DMEM, enriched with 10% (*v/v*) platelet lysate, penicillin, streptomycin, and amphotericin B. On reaching sub-confluence, the MSCs were detached from the flask with 0.05% (*w/v*) trypsin-EDTA, counted, seeded in flasks (10,000 cells/cm²) at 37 °C and 5% CO₂, and amplified. Upon reaching sub-confluence, the cells were ready for the induction of secretome production. All cells used were at passage 1 and tested for trilineage differentiation and microbiological controls.

4.1.2. Mesenchymal Stem/Stromal Cell-Derived Secretome Production

Secretome production was induced by platelet lysate starvation of MSCs. In detail, the culture medium was removed and the cells were repeatedly washed (at least three times) with PBS without Ca^{2+} and Mg^{2+} to eliminate any residual platelet lysate [48]. MSCs were then cultured in a DMEM medium without platelet lysate for 24 h, replacing the culture medium after the first 9 h. Both conditioned media (collected at 9 and 24 h, respectively) were blended; MSCs were detached with trypsin-EDTA and counted in a Burkert chamber.

4.1.3. Ultrafiltration

The collected conditioned media was firstly centrifuged at $3500 \times g$ for 10 min to eliminate cell fragments and apoptotic bodies. Subsequently, supernatants were collected, and the ultrafiltration process was performed applying a tangential flow filtration using KrosFlo® Research 2i system (Spectrum Laboratories, Milan, Italy), using Molecular Weight Cut Off (MWCO) of a 5 kDa filtration module (Spectrum Laboratories, Milan, Italy). For the ultrafiltration process, all the instrument components were sterilized to operate in aseptic conditions under a laminar flow hood. The ultrafiltration process consists of two phases, at first allowing to concentrate the sample, and then to diafilter with sterilized and ultrapure water; according to manufacturer's guidelines, during each step, the shear rate of the feed stream was kept between 2000 s^{-1} and 6000 s^{-1} , while the trans-membrane pressure index was kept at maximum 5 psi. The concentration step led to a concentration of 0.5×10^6 cell equivalents per mL. The evaluation of the process scale-up was calculated as average liters per m^2 per h:

$$\text{L/m}^2/\text{h} = \text{permeate flux (mL/min)} / \text{cartridge superficial area (m}^2\text{)} \times 0.06$$

4.1.4. Secretome Freeze-Drying

As the elective cryoprotectant, mannitol was chosen for this study, and it was mixed with the purified secretome at the concentration of 0.5% (*w/v*). The resulting solution was at first frozen at -80°C , and then a conservative freeze-drying process was adopted (Heto PowerDry PL3000) at 8×10^{-1} mbar and -50°C for 72 h. The lyophilized secretome was kept at -20°C up to the time of use (maximum 9 months). Before and after the freeze-drying process, Lyosecretome yield (mg) was established, and cell equivalents per mg were calculated by dividing the total cell number used for production and the gained milligrams of Lyosecretome.

4.2. Lyosecretome Characterization

4.2.1. Residual Humidity and Osmolarity

After the samples rebalance at room temperature, residual humidity was determined by the Coulometric Titrator HI904 (Hanna Instruments). The titration was conducted two times on each vial ($n = 3$ vials). After reconstitution of 10 mg of lyophilized product in 2 mL saline (0.9% *w/v* NaCl in water) at 37°C , the osmolarity was measured using a micro osmometer (Precision System Inc., Natick, MA, USA). The pH of the reconstituted product was measured by pH meter (Mettler-Toledo, Columbus, OH, USA).

4.2.2. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectra of Lyosecretome were obtained using a Spectrum One Perkin-Elmer spectrophotometer (Perkin Elmer, Wellesley, MA, USA) equipped with a MIRacle™ ATR device (Pike Technologies, Madison, WI, USA). The IR spectra in transmittance mode were collected in the spectral region of $650\text{--}4000 \text{ cm}^{-1}$ due to the accumulation of 64 scans with a resolution of 4 cm^{-1} .

4.2.3. Thermal Characterization

Differential scanning calorimetry (DSC) analysis measured temperature and enthalpy values with a Mettler STAR® system (Mettler Toledo, Columbus, OH, USA) equipped with

a DSC81^e Module and an Intracooler device (Jukabo FT 900) for sub-ambient temperature analysis. Firstly, the instrument was previously calibrated with Indium as a standard reference. Outcome curves were achieved recording on about 3 mg of samples in 40 μ L sealed aluminum pans with pierced lids (method: -30 – 350 $^{\circ}$ C temperature range; heating rate 10 K min^{-1} ; nitrogen air atmosphere flux 50 mL min^{-1}). Experiments were performed in triplicate.

Thermogravimetric analysis (TGA) was measured with a Mettler STAR^e system equipped with a TGA/DSC1. Firstly, the instrument was calibrated with Indium as a standard reference. Outcome curves of the mass losses were obtained recording on 3–4 mg of samples in 70 μ L alumina pans (method: 30 – 350 $^{\circ}$ C temperature range; heating rate 10 K min^{-1} ; nitrogen air atmosphere flux 50 mL min^{-1}). Experiments were performed in triplicate.

4.2.4. Morphology Investigation by Scanning Electron Microscopy (SEM)

Lyosecretome morphology has been investigated by SEM with a Zeiss EVO MA 10 (Carl Zeiss, Oberkochen, Germany). For the analysis, the samples were coated with a gold-sputter under argon.

4.2.5. Particle Size Distribution

The particle size distribution of Lyosecretome was evaluated by performing Nanoparticle Tracking Analysis (NTA) using NanoSight NS 300 (Malvern Instruments, Malvern, UK). In detail, the freeze-dried powder was resuspended in 1 mL of deionized water (1 mg/mL) and analyzed with a dilution factor of 1:10. All measurements were repeated for 6 cycles of 60 s each, using NanoSight protocols.

4.2.6. Microvesicles Surface Biomarkers by ExoView Analysis

ExoView (Alfatest, I) is an innovative test to determine the size and number of exosomes in a sample by biomarker detection. The Tetraspanin Human standard Kit with a thin layer of CD63, CD81, and CD9 was chosen, and solutions for sample dilution were supplied by the Nano View Bioscience company. The analysis method could be divided into two macro steps: Interferometric imaging that gives information about the exosome's number and exosome's size; and fluorescence analysis that offers the intensity of a positive-marker protein. Samples were prepared in triplicate and diluted according to the protocol suggested by the supplier for the tetraspanin kit (NanoView Biosciences; EV-TETRA) and incubated for 16 h on ExoView tetraspanin chips. The chips were coated with a fluorescent antibodies mixture of anti CD81, CD63, or CD9, placed on an orbital shaker at 500 rpm for 1 h, and then washed four times. Chips were transferred into a Petri plate with MilliQ water for the final washing step and, after drying with an adsorbent paper, placed onto ExoView instrument's chip holder and analyzed. The results are the average of three determinations.

4.2.7. Total Protein Quantification

At the end of the lyophilization process, Lyosecretome protein quantification was determined by using the BCA-Protein Assay Kit (Thermo Fischer Scientific, Milan, Italy) following the manufacturer's specifications. The absorbance–concentration calibration curve was generated using bovine serum albumin (BSA) standards. Working reagent solution was added to each sample and standard (ratio 1:1) and then incubated at 37 $^{\circ}$ C for 2 h before measuring the absorbance at 562 nm with a microplate reader (Synergy HT, Milan, Italy). The Lyosecretome protein amount was estimated as a plot function comparing concentration vs. absorbance obtained for the standard protein solutions, using a third-order polynomial equation, with $R^2 = 0.99$. Results are reported as μ g of protein per mg of Lyosecretome. Each sample was tested in triplicate.

4.2.8. Total Lipid Quantification

To prepare the Nile Red stock solution, the Nile Red powder was solubilized in acetone (3.14 M), avoiding light exposure, and kept at 4 °C. The stock solution was diluted 100× in PBS (pH = 7.4) before use, and 10 µL of it were incubated with 90 µL of samples. After 5 min, the relative fluorescence was calculated by Synergy HT (530/25 excitation and 645/40 emission). The Lyosecretome lipid amount was estimated as a plot function comparing concentration vs. absorbance obtained for standard phosphatidylcholine solutions, using a third-degree polynomial equation, with $R^2 = 0.99$. Results are reported as µg of lipid per mg of Lyosecretome. Each sample was tested in triplicate.

4.2.9. Sterility Test and Microbiological Control

Each batch was investigated for sterility, endotoxins, Mycoplasma, and microbiological contaminations, as described in the current version of European Pharmacopoeia. Briefly, sterility and microbial examinations were performed as indicated in EuPh 2.6.27 and 2.6.1 chapters, respectively. Furthermore, a bacterial endotoxins evaluation was carried out by the Limulus Amebocyte Lysate test (EuPh 2.6.14), a chromogenic kinetic method, and measured as an Endotoxin Unit (EU). The test was performed following the manufacturer's instructions. Finally, mycoplasma contamination was investigated by performing specific tests (NAT test, EuPh 2.6.7.).

4.3. In Vitro Potency Test

4.3.1. Elastase Inhibitory Assay

The in vitro inhibitory effect of equine Lyosecretome on the elastase enzyme was evaluated using the method previously described in the literature, with some modifications [49]. Pancreatic porcine elastase (Merck Life Science S.r.l.) was solubilized in phosphate buffer pH 6.8 (0.5 IU mL⁻¹). The substrate N-succinyl-Ala-Ala-Ala-p-nitroanilide (Merck Life Science S.r.l.) was dissolved in TRIS buffer until a final concentration of 0.41 mmol L⁻¹ was reached. All the tested concentrations (2, 5, 10, 20 mg mL⁻¹) were incubated with the enzyme for 20 min, and, consequently, the substrate was added right before reading the microplates to begin the reaction. The kinetic reaction was monitored by spectrophotometric analysis (Synergy HT) at the absorbance of 410 nm for 35 min (measurements were made every minute). The reaction mixture in the absence of sample was used as a negative control, while the epigallocatechin gallate (EGCG) (Merck Life Science S.r.l.) was used as a positive control. The absorbance value of each sample was subtracted from the absorbance of the blank mixture (sample without the enzyme and substrate). Analyses were performed in triplicate. The inhibition rate was reported as a percentage of anti-elastase activity and calculated as follows:

$$\text{Activity (\%)} = [(A_{\text{CTR}} - A_{\text{samp}}) / A_{\text{CTR}}] \times 100$$

where A_{CTR} is the negative control absorbance and A_{samp} is the Lyosecretome sample absorbance.

4.3.2. Proliferative Test

The proliferative effects of Lyosecretome were evaluated on four different equine cell lines: Chondrocytes, tenocytes, synovial fluid, and adipose tissue-derived MSCs. Tissue samples were collected at the local abattoir following the related Italian Law from three healthy 5- to 10-year-old male horses of unknown breed, ensuring the absence of viral and bacterial pathologies. AD-MSCs were isolated and expanded as described above (see paragraph 2.1.2).

Synovial Fluid Cells Collection and Expansion

The synovial fluid was collected from the metacarpophalangeal (fetlock) joint. Following trimming and disinfection, 4–5 mL of fluid were collected with a sterile syringe, transferred in a 50 mL falcon-type tube, and diluted 1:10 with sterile phosphate-buffered

saline (PBS). After accurate mixing, the tube was centrifuged at $500 \times g$ for 20 min. The cell pellet was resuspended in 2 mL of DMEM supplemented with penicillin (50 U/mL), streptomycin (25 µg/mL), and amphotericin B (2 µg/mL) and transferred in two 25 cm² culture flasks. After 48 h, the medium was changed. The attached cells were expanded until they reached about 80% confluence when they were trypsinized and expanded at a 1:3 ratio. Cells were used for further experiments at passage P3–P4.

Tenocytes Isolation and Expansion

Tissue samples of superficial digital flexor tendon (SDFT) were collected in sterility and dissected in 2–3 mm³ pieces with a scalpel blade, removing tendon sheath. The tissue was digested overnight in 1 mg/mL collagenase II (5 mL/g of tissue) at 37 °C. After digestion, the cell suspension was strained (40 µm nylon mesh) and then centrifuged at $900 \times g$ for 15 min. The cell pellet was resuspended in 2 mL of DMEM supplemented with 1% (*v/v*) penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (2 µg/mL). Cell viability was assessed by Trypan blue staining. Cells were then transferred in 25 cm² culture flasks (10,000 cm²) and expanded in DMEM at 37 °C, 5% CO₂. Upon reaching P3–P4, cells were used for further experiments.

Chondrocytes Isolation and Expansion

Cartilage tissue samples were collected from the metacarpophalangeal joint. The joint was exposed in sterility, and about 1 g of cartilage was collected with a scalpel blade in small pieces. Tissue samples were digested overnight in 5 mL of collagenase type II (0.1%, *v/v* in DMEM) at 37 °C [2]. After digestion, the cell suspension was strained (40 µm nylon mesh) and centrifuged at $180 \times g$ for 15 min. The cell pellet was resuspended in DMEM supplemented with 1% (*v/v*) penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (2 µg/mL) and then seeded with the same medium in 25 cm² culture flasks (10,000 cells/cm²). Cells were used for in vitro metabolic activity assay at passage P3–P4.

Proliferative Test by MTT

The MTT assay was performed to assess cell metabolic activity in the four different cell lines reported above (AD-MSCs, SF-MSC, tenocytes, and chondrocytes) as potential targets for the use of Lyosecretome in osteoarticular diseases. In detail, 10,000 cells/well were plated in 96-well plates in DMEM supplemented with 10% Fetal Bovine Serum (FBS); three replicates were prepared for each treatment. After 24 h, the medium was replaced with serum-free DMEM containing Lyosecretome at the concentrations of 400,000, 200,000, and 100,000 cells/well. Cells were cultured for a further 48 h, and then the MTT test was performed as previously reported [50]. Briefly, the cells' optical density (OD) after MTT treatment was measured at 570 nm and 670 nm (reference wavelength). The cell metabolic activity percentage was calculated as $100 \times (\text{OD}_{\text{sample}} / \text{OD}_{\text{control}})$. Cells cultured with DMEM containing 10% FBS were considered a positive control (ODcontrol), while the cells cultured without serum (serum-free medium) were considered a negative control. The MTT assay was repeated with four different cell preparations for each cell line.

4.4. Proteomic Investigation

4.4.1. LC-MS/MS Analysis

Two milliliters of sample were concentrated to 100 µL in a vacuum concentrator at 60 °C, and the protein concentration was assessed using the SPNTM-Protein Assay kit (G-Biosciences, St. Louis, MO, USA); 50 µg of proteins were added with 0.2% of Rapigest and held at 100 °C for 20 min. The sample was digested overnight at 37 °C by adding sequencing-grade modified trypsin (Promega, Madison, WI, USA) at an enzyme/substrate ratio of 1:50 (*w/w*). An additional aliquot of trypsin (enzyme/substrate ratio of 1:100 *w/w*) was added in the morning (4 h, 37 °C). The addition of 0.5% trifluoroacetic acid stopped the enzymatic reaction. Digested proteins were desalted using PepClean C-18 spin columns

(Pierce Biotechnology, Inc., Rockford, IL, USA), concentrated, and finally suspended in 20 μL of 0.1% (*v/v*) formic acid.

The sample was analyzed using two LC-MS/MS platforms for a total of seven technical replicates. The first LC-MS/MS system ($n = 3$) was equipped with an Eksigent nanoLC-Ultra 2D System (Eksigent, part of AB SCIEX, Dublin, CA, USA) coupled with a hybrid ion trap-Orbitrap mass spectrometer (LTQ Orbitrap XLTM ETD; Thermo Fisher Scientific, Inc., Milan, Italy). The loading pump runs in isocratic mode with 0.1% formic acid in water for 10 min at a flow rate of 3 $\mu\text{L}/\text{min}$; the gradient pump runs a 125-min gradient of 5 to 95% of eluent B (5–40% B in 110 min, 40–95% B in 15 min; eluent A, 0.1% formic acid in water; eluent B, 0.1% formic acid in acetonitrile) at a flow rate of 300 nL/min through the column (75 $\mu\text{m} \times 15 \text{ cm}$ ChromXP C18-CL 3 μm , 120 $\text{\AA}$). Eluting peptides were electrosprayed directly into a hybrid ion trap-Orbitrap mass spectrometer (LTQ Orbitrap XLTM ETD; Thermo Fisher Scientific, Inc., Milan, Italy), equipped with a nanospray ion source. The spray capillary voltage was set at 1.7 kV, and the ion transfer capillary temperature was maintained at 220 $^{\circ}\text{C}$. Full mass spectra were recorded in the positive ion mode over a 400–1600 m/z range, with a resolving power of 60,000 (full width at half-maximum). This step was followed by five low-resolution MS/MS events that were sequentially generated in a data-dependent manner on the top five ions selected from the full MS spectrum, using dynamic exclusion for the MS/MS analysis. In particular, the MS/MS scans were acquired by setting normalized collision energy of 35% on the precursor ion and, when a peptide ion was analyzed twice, applying an exclusion duration of 0.5 min.

The second LC-MS/MS system ($n = 4$) was equipped with a two-dimensional micro-high-performance liquid chromatography system (Surveyor HPLC; Thermo Fisher Scientific, Inc., San Jose, CA, USA) coupled online to an LTQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Ten microliters of peptide mixtures were concentrated and desalted online by C18 traps loaded on a 10-port valve before final separation on the capillary reversed-phase column (Biobasic-C18, 0.180 i.d. $\times 100 \text{ mm}$, 5 μm particle size, Thermo Fisher Scientific, San Jose, CA, USA). The flow rate was 100 $\mu\text{L}/\text{min}$, which was split to achieve a final flux of 2 $\mu\text{L}/\text{min}$. Peptides were separated with the following eluents: (A) 0.1% formic acid in water; (B) 0.1% formic acid in acetonitrile; the gradient profile was 5–40% B in 110 min, 40–95% B in 15 min; the flow rate on C-18 column was 1 $\mu\text{L}/\text{min}$. The peptides eluted from the C18 column were directly analyzed with an LTQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a nano-ESI source. Full MS spectra were acquired in positive mode over a 400–2000 m/z range, followed by five MS/MS events sequentially generated in a data-dependent manner on the first five most intense ions selected from the full MS spectrum (collision energy 35%) and using dynamic exclusion for MS/MS analysis.

4.4.2. Proteomic Data Processing

The experimental MS/MS spectra produced by LTQ and LTQ Orbitrap XLTM ETD mass spectrometers were matched against the *in silico* tryptic peptide sequences of the *Equus caballus* protein database retrieved from UNIPROT in March 2021 (44484 protein sequences). Data processing was performed by Discoverer 2.5 software, based on the SEQUEST HT algorithm [51]. Peptide and protein assignment was made according to specific guidelines [52]. The following criteria were used for peptide identification: Parent mass tolerance of 50 ppm and 200 ppm was set for LTQ Orbitrap XLTM ETD and LTQ, respectively; while for both peptides, fragment mass tolerance was set to 0.8 Da, respectively. Missed cleavage sites per peptide were set to 3. The percolator node was used with a target-decoy strategy to give a final false discovery rate (FDR) ≤ 0.01 (strict) based on *q*-values, considering a maximum ΔCN of 0.05. Only peptides with a minimum peptide length of 5 amino acids, confidence at “Medium” level, and rank 1 were considered. Protein grouping and strict parsimony principles were applied. To evaluate the correlation among replicate analyses, Spearman’s rank correlation was computed by JMP15.2 SAS software. To provide a rank list of the most abundant proteins in the analyzed samples,

the average Spectral count (SpC) of each protein was normalized on MW as previously reported [53]. Functional Annotation Tool of DAVID database [54] was used to characterize the most enriched molecular function (MF), biological process (BP), and cellular component categories; specifically, background = *Equus caballus*, count > 5 and $p < 0.01$ were set.

An *Equus caballus* protein–protein interaction (PPI) network was built by homology with *Homo sapiens*, as previously reported [55]. Proteins identified in at least 2 out of 7 replicate analysis were combined with the *Homo sapiens* PPI network retrieved from the STRING database [56]. Only experimentally and database-defined PPIs with a score > 0.15 and 0.35, respectively, were considered. The resulting sub-network was visualized and analyzed by Cytoscape and its plugins [57]. It was processed at the functional level using STRING Cytoscape APP [56]. Network topological analysis was performed by Centiscape2.2 Cytoscape plugin as previously reported [58]; betweenness, centroid, and bridging centralities were evaluated. A set of nodes with values above the average calculated on the whole network were defined as hubs [59]. The statistical significance of topological results was tested by considering randomized network models; they were reconstructed and analyzed by an in-house R script based on VertexSort (to build random models), igraph (to compute centralities), and ggplot2 (to plot results) libraries.

4.5. Statistical Analysis

Raw data were processed through STATGRAPHICS XVII (Statpoint Technologies, Inc., Warrenton, VA, USA). A general linear analysis of variance model (ANOVA) was generated to evaluate the data. The function was then followed by an LSD test to estimate the differences between means. Each batch was processed, considering protein and lipid content as the response variable and the batch number as the fixed factor. To evaluate cell proliferation, the cell metabolic activity was set as the response variable and the Lyosecretome concentration as a fixed factor. Statistical significance was set at $p < 0.05$.

5. Conclusions

This study provides the proof-of-principle of the feasibility of a clinical-grade injectable freeze-dried formulation containing the secretome from equine adipose tissue-derived mesenchymal stem/stromal cells. The technological production process has proven to be consistent, and a panel of tests for quality control and batch release has been defined. Lyosecretome exerts a direct activity on different musculoskeletal cell types such as tenocytes, chondrocytes, and tissue-resident MSCs; also, proteomic composition suggests a possible role of Lyosecretome for regenerative medicine applications. In detail, our findings suggest the therapeutic role in musculoskeletal diseases and wound healing of many proteins found in the equine secretome, including SLRP family members. Certainly, a more in-depth evaluation of the role of these proteins could be performed, and it will be the object of future investigations. Although extensive in vitro characterization is needed, this work paves the way for the clinical use of Lyosecretome: The proof-of-concept for manufacturing clinical-grade equine Lyosecretome has been provided, and prototypes are now available to evaluate its safety and efficacy in the treatment of horse musculoskeletal diseases.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/ph14060553/s1>, Figure S1. Functional evaluation of the protein profile characterized from equine mesenchymal stem/stromal cells. (A) Biological Processes, (B) Cellular Components, and (C) Molecular Functions enriched by considering protein secreted by equine mesenchymal stem/stromal cells. Fold enrichment was calculated by DAVID db ($p < 0.01$); Figure S2. *Equus caballus* protein–protein interaction (PPI) network reconstruction. (A) Homology between *Equus caballus* and *Homo sapiens* protein sequences. (B) Distribution of the global STRING score, (C) experiments STRING score, and (D) database STRING score found for the protein–protein interactions used to reconstruct the *Equus caballus* PPI network. Table S1. List of proteins identified by analyzing the Lyosecretome obtained by equine adipose-derived mesenchymal stem cells; Table S2. List of proteins involved in inflammatory processes and response to bacterium.

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Part II

Objective - Canine Lyo-secretome production, characterization, and preliminary *in vivo* safety evaluation

Resume – The same procedure was used to obtain canine batches of Lyosecretome from adipose tissue-derived MSCs, characterized through the same panel of quality controls. Next, *in vitro* potency tests were performed to assess proliferative effects on different cell lines. Finally, a preliminary *in vivo* safety evaluation was performed in three patients spontaneously suffering from elbow or knee osteoarthritis, treated with two intra-articular injections of Lyo-secretome resuspended in hyaluronic acid, at a 40-day interval. Clinical and orthopedic visits and an owner questionnaire were used for the follow-up.

Published article - Canine Mesenchymal Cell Lyosecretome Production and Safety Evaluation after Allogenic Intraarticular Injection in Osteoarthritic Dogs. Michela Mocchi, Elia Bari, Silvia Dotti, Riccardo Villa, Priscilla Berni, Virna Conti, Maurizio Del Bue, Gian Paolo Squassino, Lorena Segale, Roberto Ramoni, Maria Luisa Torre, Sara Perteghella and Stefano Grolli

Key aspects – The *in vitro* studies confirmed the positive effects previously shown for equine species. Furthermore, this study suggests that repeated IA injection of allogeneic Lyo-secretome is safe and does not induce clinically adverse reactions, paving the way for a complete clinical trial to assess the Lyo-secretome efficacy in the treatment of dogs with naturally occurring osteoarthritis.

Article

Canine Mesenchymal Cell Lyosecretome Production and Safety Evaluation after Allogeneic Intraarticular Injection in Osteoarthritic Dogs

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Simple Summary: Recently, mesenchymal stromal cells have been proposed as a valid approach for treating osteoarthritis diseases, mainly due to the secretion of soluble factors and extracellular vesicles known as secretome. In this paper, an injectable freeze-dried pharmaceutical powder containing canine mesenchymal stromal cells-secretome (Lyosecretome) has been formulated, and in vitro potency experiments were conducted. Furthermore, the Lyosecretome was applied in dogs affected by osteoarthritis to assess its safety. Results indicated that intra-articular injection of allogeneic Lyosecretome is safe and does not induce an adverse response, thus paving the way for future studies regarding the efficacy.

Abstract: In recent years, mesenchymal stromal cells (MSCs) have shown promise as a therapy in treating musculoskeletal diseases, and it is currently believed that their therapeutic effect is mainly related to the release of proteins and extracellular vesicles (EVs), known as secretome. In this work, three batches of canine MSC-secretome were prepared by standardized processes according to the current standard ISO9001 and formulated as a freeze-dried powder named Lyosecretome. The final products were characterized in protein and lipid content, EV size distribution and tested to ensure the microbiological safety required for intraarticular injection. Lyosecretome induced the proliferation of adipose tissue-derived canine MSCs, tenocytes, and chondrocytes in a dose-dependent manner and showed anti-elastase activity, reaching 85% of inhibitory activity at a 20 mg/mL concentration. Finally, to evaluate the safety of the preparation, three patients affected by bilateral knee or elbow osteoarthritis were treated with two intra-articular injections ($t = 0$ and $t = 40$ days) of the allogeneic Lyosecretome (20 mg corresponding 2×10^6 cell equivalents) resuspended in hyaluronic acid in one joint and placebo (mannitol resuspended in hyaluronic acid) in the other joint. To establish the safety of the treatment, the follow-up included a questionnaire addressed to the owner and orthopaedic examinations to assess lameness grade, pain score, functional disability score and range of motion up to day 80 post-treatment. Overall, the collected data suggest that intra-articular injection of allogeneic Lyosecretome is safe and does not induce a clinically significant local or systemic adverse response.

Keywords: mesenchymal stem/stromal cells; MSC-secretome; canine regenerative medicine; osteoarthritis

1. Introduction

In the last few years, mesenchymal stem cells (MSCs) have shown promise as a therapy in the treatment of musculoskeletal diseases (MSDs), including different pathological conditions such as disorders of muscle, joint, bone, nerves, and tendons [1]. The disease burden of MSDs in veterinary medicine is considerable and represents a significant threat to animal welfare worldwide, particularly in canine and equine species [2,3]. MSDs are related to various factors, such as intensive and repetitive works, sport-related trauma, ageing, and genetic background [1]. However, the MSCs ability to undergo multi-lineage differentiation has long been considered a critical feature to justify their clinical application. Currently, it is proved that the therapeutic effect of MSCs is mainly related to the secretion of a wide variety of trophic factors, encompassed under the name of secretome [4,5], playing an important paracrine activity.

MSCs secretome is composed of a combination of soluble factors (mainly cytokines and growth factors) and insoluble extracellular vesicles, including exosomes and microvesicles. The numerous different features associated with the secretome suggest that it could be used as an MSCs substitute [5] to mediate their biological activity, including anti-inflammatory and tissue regenerative properties [6]. The use of MSC-secretome as an alternative to their parental cells brings several advantages in regenerative medicine: the use of a cell-free product would reduce the unwanted immune reaction, and the tumorigenic risk related to the MSCs proliferation and differentiation capability; it is also easier to handle, store and ensure a sterile product by filtration [7–9]. We recently proposed a GMP-compliant human secretome production process that allowed us to obtain a stable and effective product named Lyosecretome (freeze-dried secretome) from human adipose tissue-derived MSCs [10–14]; this process included an ultrafiltration step for the concentration and the purification of MSCs-derived secretome, and the following lyophilization, obtaining a powder dosage form ensuring an improved long-term stability of the secretome.

In the current work, we applied the same procedure to obtain canine Lyosecretome from adipose tissue-derived MSCs, as a candidate for in vivo testing on naturally occurring diseases, thanks to the promising regenerative potential of MSCs secretome. Canine Lyosecretome was first characterized through quality controls regarding physicochemical properties, vesicles morphology and size distribution, protein and lipid content; furthermore, the requirements for the preparation of injectable pharmaceutical dosage forms were evaluated. Next, in vitro potency tests were performed to assess proliferative effects on different cell lines. Finally, the proof of principle of the safety of the canine allogenic Lyosecretome was performed by intraarticular administration to osteoarthritic dogs. As far as we know, this study is the first canine study based on MSCs secretome in naturally occurring musculoskeletal diseases. The application of Lyosecretome to animals affected by spontaneous osteoarthritis, avoiding the use of experimentally induced disease models, could provide interesting information about the safety and feasibility of an allogenic cell-free regenerative medicine approach in companion animals that share with the owner's environment and lifestyle [15], thus providing valuable clues for osteoarthritis therapy in the human counterpart.

2. Materials and Methods

2.1. Materials

The Lyosecretome production was made at an accredited facility to produce and control veterinary products for clinical use (Istituto Zooprofilattico Sperimentale Lombardia and Emilia-Romagna, IZLER, Brescia, Italy). The production protocol has been approved by the Italian Ministry of Health (Prot. n. 0000778 del 15/01/2020 7.1.2.0.0.0/17/2019-

AGD 809). Adipose-derived canine MSCs were obtained by IZLER Biobank. All reagents for cell culture, e.g., DMEM, fetal bovine serum (FBS) and antibiotics, were purchased from Euroclone, Milan, Italy. Acetone, bovine serum albumin (BSA), mannitol and phosphatidylcholine (PC) were bought from Sigma Aldrich, Milan, Italy. Epigallocatechin gallate (EGCG), N-succinyl-Ala-Ala-Ala-p-nitroanilide and pancreatic porcine elastase were purchased from Merck Life Science, Milan, Italy. Otherwise specified, all the reagents used were of analytical grade.

2.2. Preparation and Characterization of Injectable Lyosecretome Formulations

Freeze-dried MSC-secretome (Lyosecretome) was prepared and characterized following and adapting previous procedures in compliance with the current standard ISO 9001 [10–14]. Adipose-derived MSCs (AD-MSCs) were seeded into flasks at 10,000 cells/cm² and expanded until P3 with DMEM/F12 medium plus 10% *v/v* FBS, plus 1% *v/v* penicillin/streptomycin and 1% *v/v* amphotericin B at 37 °C and 5% CO₂. Secretome was obtained by culturing MSCs in DMEM/F12 without FBS (FBS starvation) for 48 h. MSCs employed accomplished the requirements for clinical use in terms of cell viability, cellular identity, genomic stability (as stated by the International Society for Cellular Therapy [16]), sterility and apyrogenicity (according to Eu. Ph. 9.0, 2.6.7). Supernatants collected from the cell culture were centrifuged at 3500 × *g* for 10 min to remove cell debris and apoptotic bodies. Supernatants were recovered and then ultrafiltered by tangential flow filtration (KrosFlo® Research 2 i system, Spectrum Laboratories, Milan, Italy) using a filtration module with a superficial area of 235 cm² and a molecular Weight Cut Off (MWCO) of 5 kDa (Spectrum Laboratories, Milan, Italy). The samples were first concentrated at 0.5 × 10⁶ cell equivalents per mL (calculated by dividing the total cell number and the concentrated mL of supernatant). Then, they were diafiltered using sterile ultrapure water as a buffer. The processed sample was then added of mannitol at a final concentration of 0.5% *w/v*, frozen at −80 °C and freeze-dried (Christ Epsilon 2–16 D LSCplus) at −50 °C and 8 × 10^{−1} mbar for 72 h. The obtained Lyosecretome was stored at −20 °C until use (6 months); each mg of Lyosecretome corresponds to 0.1 × 10⁶ cell equivalents (=total cell number/obtained milligrams of Lyosecretome).

2.2.1. Lyosecretome Characterization

Proteins and Lipids Amount

Total proteins were dosed by the BCA Protein Assay Kit (Thermo Fisher Scientific, Milan, Italy) according to the manufacturer's instructions. Briefly, the working reagent solution in a 1:1 ratio was added to each sample and incubated at 37 °C for 2 h. The optical density was measured at 562 nm with a microplate reader (Synergy HT, BioTek, United Kingdom). Standard protein solutions (BSA) were used to build a calibration curve ($R^2 = 0.99$). Total lipids were dosed by the Nile Red method, which was previously validated [10]. The Nile Red powder was solubilized in acetone at a concentration of 3.14 M and then diluted 100× in PBS. Nile Red solution was added to samples (1:9 ratio) and incubated for 5 min at room temperature. The fluorescence was measured by Synergy HT at 530/25 excitation and 645/40 emission. Standard lipid solutions (PC) were used to build a calibration curve ($R^2 = 0.99$). All analyses were done in triplicate.

EV Particle Size and Concentration

Nanoparticle Tracking Analysis (NTA, NanoSight NS 300 equipment, Malvern Instruments, Malvern, UK) was used to measure EVs' particle size and concentration into Lyosecretome. The freeze-dried powder was analyzed at room temperature at the concentration of 1 mg/mL in deionized water. By adjusting the instrument setting, raw data were processed by the NTA software v3.2 (Malvern Instruments, Malvern, UK). All analyses were done in triplicate.

Physical-Chemical Characterization

Fourier-Transform Infrared Spectroscopy (FT-IR) spectra of the Lyosecretome were evaluated between 650 and 4000 cm^{-1} spectral regions with a resolution of at least 4 cm^{-1} . A Spectrum One Perkin-Elmer spectrophotometer (Perkin Elmer, Wellesley, MA, USA) arranged with a MIRacle™ ATR device (Pike Technologies, Madison, WI, USA) was exploited. All measurements were done in triplicate. Differential Scanning Calorimetry (DSC) analysis was performed with a Mettler STAR^e system (Mettler Toledo, Columbus, OH, USA) equipped with a DSC821 e Module and an Intracooler device (Jukabo FT 900) (JULABO GmbH, Seelbach, Germany) for sub-ambient temperature analysis. The instrument, after calibration with Indium as a standard, was used on samples; the curves were recorded on about 3 mg in 40 μL sealed aluminium pans with pierced lid. The following parameters were considered: temperature range -30 – 250 $^{\circ}\text{C}$; heating rate 10 K min^{-1} ; nitrogen air atmosphere flux 50 mL min^{-1} . Each experiment was performed in triplicate. Thermo Gravimetric Analysis (TGA) was performed with a Mettler STAR^e system (Mettler Toledo, Columbus, OH, USA) arranged with a TGA/DSC1 (Mettler Toledo, Columbus, OH, USA). The instrument was previously calibrated with Indium as a standard reference; then, the curves were recorded on about 4 mg of samples in 70 μL alumina pans. The following parameters were considered: temperature range 30 – 300 $^{\circ}\text{C}$; heating rate 10 K min^{-1} ; nitrogen air atmosphere flux 50 mL min^{-1} . Each experiment was performed in triplicate.

Sterility and Apyrogenicity

Sterility, endotoxins, mycoplasma and other microbiological contaminations were evaluated on a representative sample using the same tests applied to ensure the sterile conditions of the starting cells. In detail, for microbial control, the samples were examined through a microbiology test according to the provisions of the European Pharmacopoeia (EuPh 2.6.2). Apyrogenicity was ensured by quantitatively detecting bacterial endotoxins using the Limulus Amebocyte Lysate (LAL) test (EuPh 2.6.14) and measured as Endotoxin Unit (EU). Possible mycoplasma contamination was detected by carrying out specific tests (NAT test) following the provisions of European Pharmacopoeia (EuPh 2.6.7).

2.3. In Vitro Potency and Efficacy Tests

2.3.1. Specimen Collection

Adipose tissue, tendon, and cartilage tissue samples were collected from animals at the local Veterinary Teaching Hospital. All animal owners were aware of the study and signed an informed consensus, and the protocols were approved by the Italian Ministry of Health (Prot. n. 0000778 del 15/01/2020 7.1.2.0.0.0/17/2019-AGD 809). All the animals were subjected to a complete clinical examination, blood count (CBC), and serum biochemistry analysis. Tendons and cartilage were collected two to six hours after the death from the knee joint of animals who died of natural causes and were subjected to necropsy. Patients affected by infectious diseases were excluded.

2.3.2. MSCs Isolation from Adipose Tissue, Tendon and Cartilage

Once collected, each type of tissue was washed in 70% ethanol and stored in phosphate-buffered saline (PBS) containing penicillin (50 U/mL), streptomycin (20 $\mu\text{g/mL}$), and amphotericin B (2.5 $\mu\text{g/mL}$) for a maximum of 2h before being processed. Then, 1–1.5 g of tissue were fragmented in 0.3–0.5 cm^3 pieces in a Petri dish with scalpels and sterile pliers. The fragments were transferred to a 15 mL conical centrifuge tube containing a solution of 0.1% *w/v* collagenase type I, prepared in DMEM, supplemented with 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 2.5 $\mu\text{g/mL}$ amphotericin B, at a ratio of 5 mL of medium per gram of minced tissue. The samples were enzymatically digested in a water bath at 37 $^{\circ}\text{C}$, in moderate agitation, for 1 h for adipose tissue and 2 h for tendon and cartilage fragments. The solution was then filtered with a nylon filter (mesh 100 μm) and centrifuged at $190\times g$ for 15 min. The pellet obtained was followed by resuspension in 2 mL of maintenance medium (mDMEM), consisting of DMEM supplemented with 10% *v/v* FBS, penicillin

100 U/mL, streptomycin 100 µg/mL, amphotericin B 2.5 µg/mL, and then seeded in 25 cm² cultures flasks. The cells were maintained in an incubator at 37 °C in a 5 % CO₂ atmosphere, renewing the medium every 72 h. Once 80% confluence was reached, the cells were detached with 0.05% Trypsin-EDTA in PBS and expanded to P3–P4 when used for the experiments described below.

2.3.3. Cell Metabolic Activity Evaluation

The cytocompatibility and proliferation ability of Lyosecretome were evaluated on three different cell lines: canine MSCs, canine tenocytes and canine chondrocytes. Cells were seeded in 96-well plates at 10,000 cells/well for 24 h and treated with increasing doses of Lyosecretome (50,000, 100,000 and 200,000 cell equivalents/well) in a serum-free medium. Cells not treated with Lyosecretome or treated with 10% *v/v* FBS were considered negative and positive control. After 48 h, an MTT test was performed. The absorbance was measured by a microplate reader (Victor Nivo, Perkin Elmer, Waltham, MA, USA) at 570 nm and 670 nm (reference wavelength), and the metabolic activity percentage was calculated as:

$$\text{Cell metabolic activity (\%)} = 100 \times (\text{Abs}_{\text{sample}} / \text{Abs}_{\text{positive control}}). \quad (1)$$

2.3.4. Anti-Elastase Activity

The suppressive effect of Lyosecretome on pancreatic porcine elastase was assessed *in vitro*. Briefly, the enzyme was solubilized in phosphate buffer pH 6.8 at 0.5 IU mL⁻¹. Simultaneously, the substrate consisting of N-succinyl-Ala-Ala-Ala-p-nitroanilide was dissolved at a final concentration of 0.41 mmol L⁻¹ in TRIS buffer. Increasing concentrations of Lyosecretome (2, 5, 10, 20 mg mL⁻¹) were incubated at room temperature with the enzyme for 20 min; then, the substrate was added, and the kinetic reaction was monitored by spectrophotometric analysis (Synergy HT) at the absorbance of 410 nm for 35 min (one measurement/minute). The reaction mixture in the absence of Lyosecretome was used as a negative control, while EGCG at 7.2 mg/mL was used as a positive control. Analyses were performed in triplicate, and the percentage anti-elastase activity (%) was calculated as:

$$\text{Anti-elastase activity (\%)} = [(A_{\text{CTR}} - A_{\text{samp}}) / A_{\text{CTR}}] \times 100 \quad (2)$$

where A_{CTR} is the optical density of the negative control, and A_{samp} is the optical density of the Lyosecretome sample.

2.4. *In Vivo* Safety Evaluation

In vivo safety of Lyosecretome was evaluated on dogs affected by naturally occurring osteoarthritis (OA).

2.4.1. Ethical Concerns

The clinical trial has been approved by the Italian Ministry of Health (Prot. n. 0000778 del 15/01/2020 7.1.2.0.0.0/17/2019-AGD 809). The clinical experimentation has also been approved by the ethical committee of Istituto Sperimentale della Lombardia ed Emilia Romagna (IZSLER, n. 1/2019, 21 March 2019).

2.4.2. Animal Recruiting and Inclusion/Exclusion Criteria.

The current study enrolled 5 client-owned dogs presenting bilateral elbow or knee osteoarthritis (Table 1). The animals were treated with two intra-articular injections of Lyosecretome at 40-days intervals. The owners of all the animals involved in the study signed a written donor consent after being notified of the relevant project information. For the enrollment, the patients underwent a complete clinical examination, complete blood count and serum biochemistry analysis to exclude other pathologies, followed by an orthopaedic examination to confirm and grade the OA. Details on the different clinical cases are reported in the Supplementary materials (Section S1, Figures S1–S3).

Table 1. Summary of cases included in this study regarding the breed, age, gender, weight and affected joint.

Animal	Breed	Age (Years)	Gender	Weight	Joint
1	Labrador	8	F	35	Knee
2	Labrador	9	F	35	Elbow
3	Golden retriever	5	M	34	Elbow
4	Labrador	3	M	32	Elbow
5	Labrador	9	M	30	Elbow

This study evaluated the following orthopaedic parameters: lameness, pain, functional disability, range of motion, and OA grade (Tables 2–4). A disease score was obtained by adding scores for each of these parameters, ranging from grade 1 to 5 (Table 5). Only Grade 5 dogs were enrolled in this study.

Table 2. Overview of the score systems used for the orthopaedic examination.

Parameter	Score	Definition
Lameness	1	Stands, walks and trots normally
	2	Stands normally, slightly painful gait when trotting
	3	Stands normally, slightly painful gait when walking
	4	Stands normally, evident painful gait when walking
	5	Stands abnormally, evident painful gait when walking
Pain	1	None
	2	Mild signs
	3	Moderate signs
	4	Severe signs
	5	The dog will not allow palpation
Functional disability	1	Normal
	2	Slightly stiff
	3	Stiff
	4	Very stiff, unwilling to walk
	5	Need assistance to walk
Range of motion	1	No limitation of movement or crepitus
	2	10 to 20% decrease in range of motion, no crepitus
	3	10 to 20% decrease in range of motion with crepitus
	4	20 to 50% decrease in range of motion
	5	More than 50% decrease in range of motion

Table 3. The Kellgren–Lawrence grading of osteoarthritis for the knee joint.

OA	Grading	Radiographic Features
Absent	0	No abnormalities
Doubtful	1	Minute osteophytes
Minimal	2	Definite osteophytes
Moderate	3	Diminished joint space
Severe	4	Greatly diminished joint space + sclerosis of the subchondral bone

Table 4. International Elbow Working Group (IEWG) guidelines for elbow osteoarthritis.

OA	Grading	Radiographic Features
Absent	0	Normal elbow joint, no evidence of incongruency, sclerosis, or arthrosis
Mild	1	Presence of osteophytes < 2 mm high, sclerosis of the base of the coronoid process—trabecular pattern still visible
Moderate	2	Presence of osteophytes of 2–5 mm high, obvious sclerosis (no trabecular pattern) of the base of the coronoid processes
Severe	3	Osteophytes of over 5 mm found anywhere in the joint

Table 5. Disease score was obtained by adding the scores of all the parameters evaluated in this study.

Sum of Scores	Disease Score
0–2	1
3–6	2
7–10	3
11–14	4
15–20	5

2.4.2. Pre-Treatment and Post-Treatment Evaluations

A full orthopaedic examination was conducted with X-rays under sedation to assess the baseline severity of OA in each animal. Radiographic signs of OA were evaluated using the Kellgren–Lawrence scale for the knee joint [17] and the International Elbow Working Group (IEWG) guidelines for the elbow joint [18]. The OA was categorized as shown in Tables 3 and 4. The orthopaedic examination of the animals included the assessment of lameness grade, pain score, functional disability score and range of motion (ROM) on day 0 (t0) and 40 days after each intra-articular treatment (t40 and t80). In detail, to assess the lameness score, gait was observed at stand, walk and trot and recorded by video, and lameness was categorized from grade 1 to 5 (Table 2) [19]. Each joint was then manipulated for pain and functional disability, and the ROM was assessed with an orthopaedic goniometer, giving a score from 1 to 5 to each parameter (Table 2) [17,19].

The follow-up included an owner questionnaire (Table 6) containing the validated Helsinki chronic pain index (HCPI) for up to 80 days (t0, t2, t4 ... t20, t40, and t80). The questionnaire inquired about the dog's general condition (body temperature, appetite) and local reactions in the treated joints (heat, pain, and joint swelling). Owners were also asked to report any abnormal response of the patient or NSAID treatment needed. Chronic pain signs were assessed with the HCPI that contained questions regarding the dog's mood, lameness, and willingness to move, play, and jump. The HCPI included 11 questions, whose answers were given by a score of 0 to 4, where 0 and 1 indicate normal behaviour and movement, while 2, 3, and 4 indicate pain with increasing severity [20–22]. The owner questionnaire based on HCPI and additional information was used to assess animal welfare before the treatment and to collect local or systemic adverse reactions, providing information about the safety of the treatment.

Table 6. Questionnaire submitted to the owners. The questionnaire is based on Helsinki chronic pain index (HCPPI), with additional information collected to evaluate local and systemic adverse reactions reported by the animal owners.

Question Asked	0 Points	1 Point	2 Points	3 Points	4 Points
D2. Pain upon right joint palpation	Absent	Mild	Moderate	Severe	Does not allow palpation
D3. Pain upon left joint palpation	Absent	Mild	Moderate	Severe	Does not allow palpation
D4. Swelling upon right joint palpation	Absent	Mild	Moderate	Severe	Does not allow palpation
D5. Swelling upon left joint palpation	Absent	Mild	Moderate	Severe	Does not allow palpation
D6.1 Heat in the right joint	No warmer than contralateral limb	Mildly warmer	Moderately warmer	Markedly warmer	
D6.2 Heat in the left joint	No warmer than contralateral limb	Mildly warmer	Moderately warmer	Markedly warmer	
D7. Rate your dog's mood	Very alert	Alert	Neither alert nor indifferent	Indifferent	Very indifferent
D8. Rate your dog's willingness to participate in play	Very willing	Willing	Reluctantly	Very reluctantly	It does not play at all
D9. Rate your dog's complaining and groaning	Never	Hardly ever	Sometimes	Often	Very often
D10. Rate your dog's willingness to walk	Very willing	Willing	Reluctantly	Very reluctantly	It does not walk at all
D11. Rate your dog's willingness to trot	Very willing	Willing	Reluctantly	Very reluctantly	It does not trot at all
D12. Rate your dog's willingness to gallop	Very willing	Willing	Reluctantly	Very reluctantly	It does not gallop at all
D13. Rate your dog's willingness to jump (e.g., into a car, onto a sofa)	Very willing	Willing	Reluctantly	Very reluctantly	It does not jump at all
D14. Rate your dog's ease in lying down	With great ease	Easily	Neither easily nor difficultly	With difficulty	With great difficulty
D15. Rate your dog's ease in rising from a lying position	With great ease	Easily	Neither easily nor difficultly	With difficulty	With great difficulty
D16. Rate your dog's ease of movement after long rest	With great ease	Easily	Neither easily nor difficultly	With difficulty	Very often/always difficulty
D17. Rate your dog's ease of movement after major activity or heavy exercise	With great ease	Easily	Neither easily nor difficultly	With difficulty	Very often/always difficulty
D18. Dog's appetite	Normal	Capricious	Decreased	Markedly decreased	It does not eat at all
D19. Dog's body temperature	Normal	Fever			
D20. Use of analgesic/anti-inflammatory drugs	No	Yes			
D21. Abnormal post-treatment reactions	None	Physical tiredness	Physical tiredness + joint pain/swelling	Increased lameness	Does not stand and walk at all

2.4.3. Intra-Articular Injection of Lyosecretome

After examination, dogs were sedated, and the skin over the joint was prepared for a sterile invasive procedure. Two product stocks were prepared by suspending Lyosecretome (20 mg corresponding to 2×10^6 cell equivalents) or mannitol (placebo) in 1 mL hyaluronic acid (Athenavis 1%, Ibn Savio, Pomezia, Italy), containing 20 mg of sodium hyaluronate, 800–1300 KDa, in 2 mL_i. For each animal, the right joint was infiltrated with the same stock and the left joint with the other stock, both times. Both the veterinarian and the owner were unaware of which treatment the joint received. In detail, a 20-gauge needle was introduced into the joint space, synovial fluid was aspirated, and the product was injected through the same needle and a syringe of 2.5 mL. At the end of the infiltration, the joints were subjected to slight flexion-extension movements to promote the spread of the product. After the procedure, the patient was sent home, prescribing rest for the first two days, resuming regular activity from the third day. The use of any anti-inflammatory medication was prohibited during the entire course of the 180 days study except in exceptional circumstances. The orthopaedic evaluation and the owner questionnaire were performed to evaluate the response to treatment and compare it with the initial clinical condition.

2.5. Statistical Analysis

STATGRAPHICS XVII (Statpoint Technologies, Inc., Warrenton, VA, USA) program was used to analyze raw data producing a general linear analysis of variance model (ANOVA). The function was then followed by an LSD test to estimate the statistical differences between means. In detail, each batch was evaluated as the variable response protein and lipid content, and the batch number was assumed as the fixed factor. The cell metabolic activity was set as the response variable and the Lyosecretome concentration as a fixed factor to evaluate cell proliferation. The anti-elastase activity data were elaborated considering the batch, concentration, and time as fixed factors, and the activity % as the response variable. Statistical significance was determined at $p < 0.05$.

3. Results and Discussion

Injectable Lyosecretome (freeze-dried secretome) formulations have been prepared by standardized processes under ISO9001 clinical grade. Overall, three batches were produced starting from three different cell lines, as summarized in Table 7.

Table 7. Lyosecretome batch report.

Batch/Cell Line	Total Cell Number ($\times 10^6$)	Cell Viability (%)
1	190	99
2	96	98
3	92	99

After ultrafiltration to concentrate and purify the cell culture supernatant, the product was added to the cryoprotectant and freeze-dried. A filtering module with a 5 kDa molecular weight cut-off was used to retain in the ultrafiltrate both extracellular vesicles (EVs) and proteins with weight above the cut-off. The use of this membrane size was justified by preliminary investigations, demonstrating a higher immunomodulatory potency in 5 kDa ultrafiltrate (containing both EVs and soluble factors) instead of the 300 kDa ultrafiltrate (containing only EVs) [10]. Furthermore, Bari et al. [11] demonstrated that the concentration of IL-6 was higher in samples prepared by ultrafiltration rather than ultracentrifugation. Since IL-6 is a key player in the immunomodulatory features of the MSCs secretome, the large amounts of IL-6 discarded in the ultracentrifugation procedure suggest ultrafiltration is an effective procedure to prepare EV fraction. Similarly, higher anti-elastase activity was demonstrated for the whole secretome and attributed to the large amount of Alpha-1-Antitrypsin, the main inhibitor of neutrophil elastase, in the soluble fraction of the secretome [14]. Accordingly, Mitchell and colleagues found that EVs and

soluble molecules act synergistically to promote tissue regeneration [23], supporting low cut-off ultrafiltration as a valuable process to prepare MSCs secretome.

The pharmaceutical quality of Lyosecretome was defined by setting up a complete characterization of total protein and lipid content, physical-chemical properties, and particle size. The amount of proteins and lipids for each batch produced is reported in Table 8. It is worth noting that no systematic error influenced the calibration for both assays as the intercept of the curve equation is not statically significant, and the plot of the residuals had an ordinary distribution of the error (data not shown). Overall, the yield of each batch is different, meaning that the cell line and, more in general, the variability within the same species can strongly influence the secretome composition in terms of protein and lipid quantity ($p < 0.05$). Maximum protein production was obtained in batch n. 1; by contrast, lipid amount is higher in batch n. 2. This aspect underlines that there is no correlation between the two assays, probably depending on cell density at the starvation time that can influence secretome production.

Table 8. Total protein and lipid content in Lyosecretome batches; mean values $\pm$ standard deviation, $n = 3$. Different letters (a, b and c) indicate a significant difference between groups of the same column ($p < 0.0001$).

Batch n.	$\mu\text{g Proteins/mg Lyosecretome}$	$\mu\text{g Lipids/mg Lyosecretome}$
1	79.5 ± 0.6^a	18.9 ± 0.5^a
2	28.6 ± 1.5^b	5.7 ± 0.1^b
3	25.9 ± 0.1^c	15.5 ± 0.3^c

The physical-chemical characterization confirmed the simultaneous presence of both proteins and lipids in the Lyosecretome. In detail, FTIR spectra revealed low-intensity bands at around 1653 cm^{-1} and 1547 cm^{-1} (amide I C=O stretching vibrations and amide II N-H bending vibrations of the peptide groups, respectively), absorbance bands at about 1457 cm^{-1} and 1377 cm^{-1} (CH_2 and CH_3 groups) and 1260 and 880 cm^{-1} (due to the stretching vibrations typical of phospholipids, triglycerides and cholesterol esters, and to the vibrations bands of mannitol) [10,11]. By DSC and TGA analysis, it was instead confirmed that the lyophilization process occurred successfully (data not shown).

Lyosecretome particles' size distribution and characterization were evaluated using NTA technology. In detail, the diameter ranges between $190\text{--}230\text{ nm}$, matching the choice to consider both exosomes ($40\text{--}120\text{ nm}$) and microvesicles ($250\text{--}1000\text{ nm}$). Furthermore, a heterogeneous population is shown by means analyzing curve, whereas d_{10} is set between $106\text{--}122\text{ nm}$, d_{50} is set between $157\text{--}180\text{ nm}$, and d_{90} is set between $316\text{--}400\text{ nm}$. Thanks to this technique, the particle concentration per mL was estimated, ranging between 1.86×10^8 and 2.60×10^8 particle/mL (Table 9 and Figure 1).

Table 9. Particle size distribution and concentration of batch 1, 2, 3 (mean values $\pm$ standard deviation, $n = 3$).

Batch n	Mean (nm)	Mode (nm)	d_{10} (nm)	d_{50} (nm)	d_{90} (nm)	Concentration Particle /mL
1	231.6 ± 8.4	143.6 ± 11.7	122.3 ± 4.1	180.6 ± 5.5	403.5 ± 20.3	$1.86 \times 10^8 \pm 6.37 \times 10^7$
2	189.1 ± 2.7	109.7 ± 3.7	106.2 ± 2.7	157.5 ± 2.8	316.6 ± 1.8	$2.60 \times 10^8 \pm 1.35 \times 10^7$
3	197.1 ± 6.0	148.0 ± 15.4	116.7 ± 1.3	171.6 ± 6.5	319.7 ± 16.6	$2.12 \times 10^8 \pm 1.06 \times 10^6$

Finally, since the Lyosecretome production was aimed at in vivo application, to guarantee the quality of the final product, microbiological tests were conducted before and after lyophilization, thus, on both secretome solution and lyophilized secretome (Lyosecretome) powder. Analyses were performed at a certified institute (IZSLER, Brescia, Italy), and the sterility conditions were ascertained in the same establishment. The results proved the absence of any mycoplasma and bacterial endotoxin level in each examined sample (data not shown).

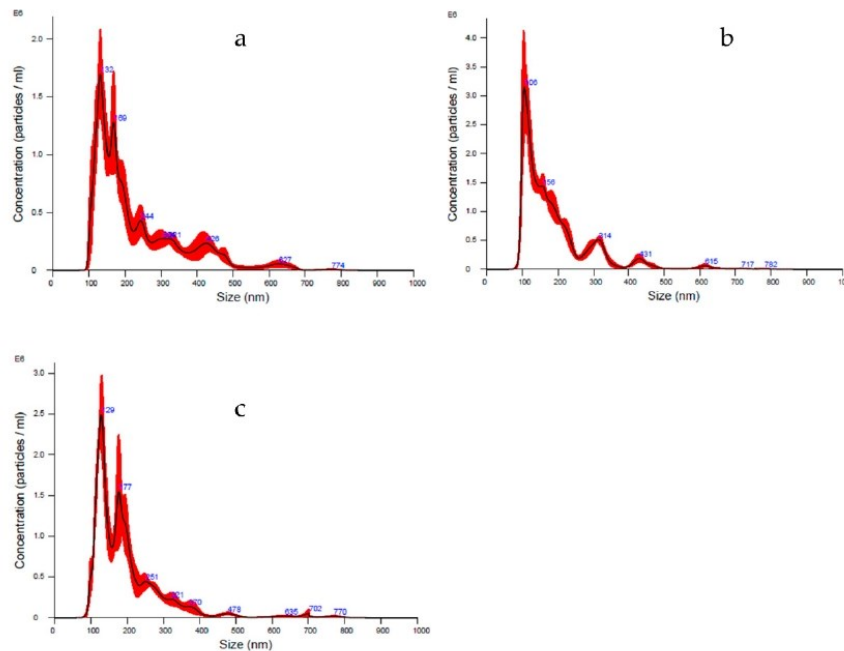


Figure 1. NTA averaged curves as particles concentration and size distribution of batch 1 (a), 2 (b), and 3 (c), respectively. The averaged curve was generated by processing six cycles of 60 s each per batch. Concentration is indicated as mean value $\pm$ standard deviation ($n = 3$). The red part shows the $\pm$ standard error of the mean values ($n = 3$).

Regarding the *in vitro* efficacy, Lyosecretome proliferation potency was assessed by MTT assay. Canine tenocytes, chondrocytes and adipose tissue-derived MSCs were chosen as target cells, the latter because the tissues resident MSCs can mediate tissue regeneration [24]. Lyosecretome stimulated cell metabolic activity in a dose-dependent trend for all cell types; in detail, the treatment with higher Lyosecretome concentrations reached almost 85% of cell metabolic activity compared to 10% FBS supplemented cultures. As shown in Figure 2, the dose-dependent effect was evaluated up to 200,000 cell equivalent/well. The effect of Lyosecretome treatment was statistically different for the three cell types ($p < 0.0001$).

The anti-elastase activity of canine Lyosecretome demonstrated a dose-dependent trend for all the tested concentrations of 2, 5, 10 and 20 mg/mL (Figure 3). The assay consists of an enzymatic reaction which was evaluated for up to 40 min. At the higher concentration of 20 mg/mL, the activity reached 85%, considering Epigallocatechin gallate as a positive control due to its inhibition of elastase activity.

Despite this study has not performed a qualitative analysis of protein, RNA, and lipid contents of canine Lyosecretome, proteomic analyses of equine [2] and human [10] Lyosecretome revealed proteins involved in controlling the inflammatory pathways activated in osteoarthritic joints and proteins related to cartilage biology. This suggests using Lyosecretome as a substitute for cells in treating musculoskeletal system diseases [25]. Furthermore, microvesicles are considered safer both from the point of view of immuno-reactivity and for the lower potential risks compared to the administration of *in vitro* expanded cells [26]. Still, before testing Lyosecretome efficacy, the *in vivo* safety must be evaluated. In this regard, this study enrolled five dogs affected by bilateral OA to test Lyosecretome for clinical use. One dog was excluded from the beginning for recent nonsteroidal anti-inflammatory drugs (NSAIDs) treatment (Animal 5, Table 1), and another dog was excluded before the

second administration of the treatment for a recurrent skin infection that could negatively affect the intra-articular treatment (Animal 4, Table 1).

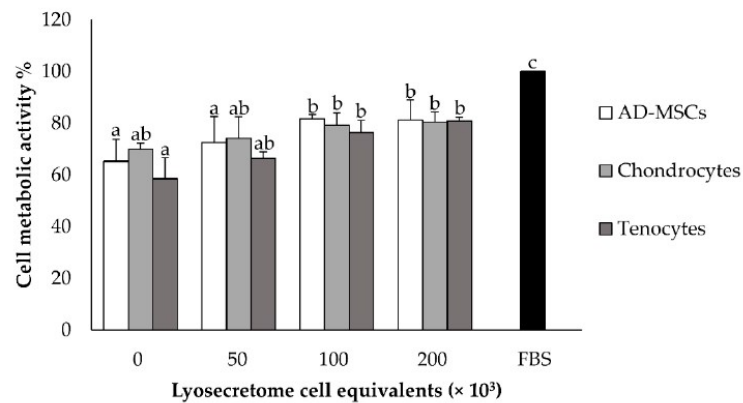


Figure 2. The dose-dependent trend of cell metabolic activity on AD-MSCs, chondrocytes and tenocytes tested at the Lyosecretome concentrations of 50, 100, 200 cell equivalents $\times 10^3$ /well. 10% v/v FBS was considered positive control, while culture media without Lyosecretome and FBS were negative control (0). Multifactor ANOVA, mean values $\pm$ LSD ($n = 3$). Different letters (a, b and c) indicate a significant difference between the means ($p < 0.05$), while the same letters indicate no significant differences ($p > 0.05$).

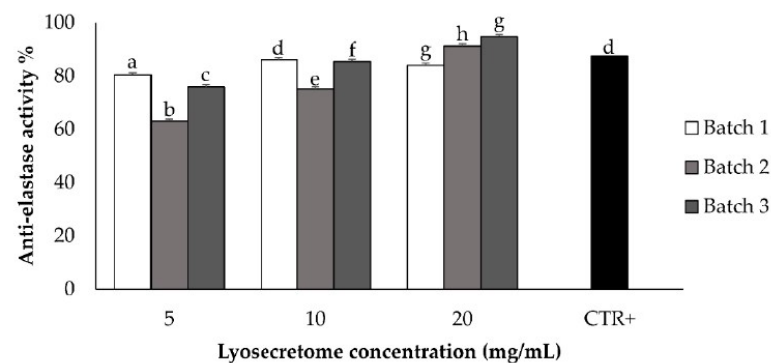


Figure 3. The anti-elastase activity was tested for the three batches at the concentrations of 2, 5, 10 and 20 mg/mL of Lyosecretome. A dose-dependent trend was obtained. CTR+ was epigallocatechin gallate at a concentration of 7.2 mg/mL in deionized water. Multifactor ANOVA, mean values $\pm$ LSD ($n = 3$). Different letters (a–h) indicate a significant difference between the means ($p < 0.05$), while the same letters indicate no significant differences ($p > 0.05$).

Lyosecretome and a placebo, consisting of mannitol (used in Lyosecretome formulation as a stabilizer), were resuspended in hyaluronic acid and injected in each animal's right and left joint, respectively. The choice to treat both affected joints with hyaluronic acid, whose lubricating properties support its widespread use in treating OA in dogs [27], was made to ensure effective therapy in the control of clinical symptoms for patients enrolled in the study. The data collected with the questionnaire (Table 6) and reported in Table S1 have been analyzed to evaluate the onset of undesired effects following therapy (see Supplementary materials, Figures S4 and S5). No systemic adverse reactions were observed, even after the second administration. After each treatment, short-time side effects were observed for all the patients' as reluctance to trot or gallop. However, these adverse effects resolved

within two days without the need for additional treatment. Since the administration of Lyosecretome (in the right joint) and placebo (in the left joint) co-occurred, it was impossible to attribute the observed symptoms to one of the two treatments. Similar side effects were also observed by Lee et al. [28]; the authors reported that following hyaluronic acid intraarticular injection, a dog showed a mild systemic inflammatory reaction, and two dogs showed non-weight-bearing lameness.

Only patient 3 was affected by swelling at the level of both the right and left joints (See Supplementary Figure S2) accompanied by complaining and groaning and not ease of movement for both lying down and moving after a long rest period. Thus, patient 3 likely showed an adverse reaction that was not directly attributable to the administration of Lyosecretome, as the same response was evidenced even when administering the placebo. Furthermore, the reported effects are commonly noticed immediately after intra-articular injections when animals' lameness score is enhanced [29,30]. Finally, regarding the clinical data, the examination by the veterinary practitioners, performed at days 40 and 80 after the first treatment, were used to assess the possible onset of unexpected symptoms after therapy administration. No significant results were observed in terms of lameness and pain worsening.

In conclusion, given the limited number of enrolled patients, the present work does not provide any direct evidence of the efficacy or safety of the Lyosecretome in the treatment of canine OA. Nevertheless, it is the proof-of-concept for veterinary clinical-grade MSC-secretome production; moreover, the feasibility of two consecutive allogeneic Lyosecretome intraarticular administration in osteoarthritic dogs is proved, without evidence of adverse reactions. Therefore, this paper represents the premise for patient recruitment in a safety/efficacy clinical trial, with a proper number of cases.

Supplementary Materials: The following are accessible online at <https://www.mdpi.com/article/10.3390/ani11113271/s1>, Table S1. Data collected from the questionnaire. File S1: History and clinical findings of the three dogs treated in the study. Figure S1. Cranio-caudal radiographic projection of the knee of Animal 1. Figure S2. Medio-lateral radiographic projections of the elbow of Animal 2. (A) Maximally flexed view of the right limb. (B) Maximally flexed view of the left limb. Figure S3. Medio-lateral radiographic projections of the elbow of Animal 3. (A) Maximally flexed view of the right limb. (B) Maximally flexed view of the left limb. Figure S4. Scores for questions 11 (A), 12 (B) and 21 (C) as a function of time. The treatment (Lyosecretome or placebo) was administered on day 0 and day 40 (circled). Multifactor ANOVA, mean values $\pm$ LSD ($n = 3$). * $p < 0.05$ vs. $t = 0$; # $p < 0.05$ vs. $t = 2$. Figure S5. Scores for questions 4 (A), 5 (B), 9 (C), 14 (D) and 16 (E) for each participant. Multifactor ANOVA, mean values $\pm$ LSD ($n = 3$). Different letters (a-h) indicate a significant difference between the means ($p < 0.05$), while the same letters indicate no significant differences ($p > 0.05$).

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Institutional Review Board Statement: The lyosecretome production protocol and the clinical investigation have been approved by the Italian Ministry of Health (Prot. n. 0000778 del 15/01/2020 7.1.2.0.0/17/2019—AGD 809). The clinical experimentation has also been approved by the ethical committee of Istituto Sperimentale della Lombardia ed Emilia Romagna (IZSLER, n. 1/2019, 21 March 2019).

Data Availability Statement: The data presented in this study are contained within the article.

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Part III

Objective – Preliminary safety and efficacy evaluation of Lyo-secretome intra-articular injections in canine and equine osteoarthritic patients

Resume – The present study represents the second part of the project, in which further batches of canine and equine batches of Lyo-secretome were prepared and characterized by following the GMP-compliant process described in the previous papers. Since no published data are available regarding MSCs-derived cell-free products in equine naturally occurring osteoarthritis, 5 horses with spontaneous fetlock OA were treated with double injections of different doses of Lyo-secretome to assess preliminary safety data. Furthermore, 21 dogs were enrolled to evaluate both safety and efficacy. Clinical evaluations, orthopedic visits, and owner questionnaires have been used to monitor the patients during the 180-day follow-up.

Article in preparation - Mesenchymal Stromal Cells Freeze-Dried Secretome (Lyo-secretome) for the treatment of joint diseases in dogs and horses. Priscilla Berni, Maurizio Del Bue, Virna Conti, Valentina Andreoli, Roberto Ramoni, Elia Bari, Maria Luisa Torre, Maurizio Rinaldi, Silvia Dotti and Stefano Grolli

Key aspects - The feasibility of two consecutive allogeneic Lyosecretome intraarticular administration in osteoarthritic patients is proved, without evidence of systemic adverse reactions. Although mild to moderate joint flare was observed in both species, the local reactions were self-limiting and in line with the results presented in other clinical studies on intra-articular injections in animals suffering from spontaneous OA, with no differences from the control group. Regarding the efficacy in canine patients, our study revealed mild improvement following the two treatment injections in both groups, without highlighting one treatment to be superior. A proper number of cases must be reached before drawing any conclusion in terms of the efficacy of this treatment.

Article in preparation

Mesenchymal Stromal Cells Freeze-Dried Secretome (Lyosecretome) for the treatment of joint diseases in dogs and horses

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SIMPLE SUMMARY

Among the species, dogs, and horses are the most deeply investigated in regenerative veterinary medicine, and among the different diseases, musculoskeletal disorders, such as osteoarthritis, are commonly found in clinical practice and represent the most studied in this field. Mesenchymal Stromal Cells (MSCs)-derived products seem to be a promising regenerative approach for treating chronic and degenerative disorders. In this paper, a freeze-dried form of secretome (Lyo-secretome) derived from adipose tissue MSCs was produced and characterized following GMP-compliant procedures, and a preliminary safety and efficacy evaluation was assessed after a double intra-articular injection of Lyo-secretome in dogs and horses spontaneously suffering from osteoarthritis.

ABSTRACT

MSCs have become an important biological alternative for the treatment of musculoskeletal disorders in both veterinary and human patients, due to their promising anti-inflammatory and pro-healing properties. Among MSCs-derived products, cell-free therapies based on MSCs-secretome or extracellular vesicles have gained popularity in recent years, since they offer similar biological effects

associated with several practical advantages when compared to the cell population. Cell-free products are indeed easier to manipulate, characterize, and store for the long-term, and they are ready to use even for the treatment of acute conditions. In this paper, a freeze-dried pharmaceutical powder containing MSCs-secretome (Lyo-secretome) has been produced for both canine and equine species. Lyo-secretome batches were characterized in terms of quantitative and qualitative analysis, and a panel of sterility and microbiologic tests was performed on the final injectable product. A double-blind, randomly assigned, controlled clinical trial was performed collecting preliminary safety and efficacy data of a double intra-articular injection of Lyo-secretome resuspended in Hyaluronic acid, in dogs and horses with naturally occurring osteoarthritis. Results indicated that the double intra-articular injection of allogeneic Lyosecretome is safe and does not induce systemic adverse response for both species. A mild to moderate joint flare was observed in some patients after the injection, although they were self-limiting and not correlated to the treatment, since no differences were found with the control group (hyaluronic acid). Regarding the efficacy in canine patients, our study revealed mild clinical improvement following the two injections in both treatment and control groups, without highlighting one treatment to be significantly superior, even if a positive tendency in terms of lameness and pain score, as well as an improvement in the Helsinki Chronic Pain Index score was observed in the Lyo-secretome group. A proper number of cases must be reached before drawing any conclusion in terms of the efficacy of this treatment.

Keywords: mesenchymal stromal cells; secretome; dog; horse; osteoarthritis; intra-articular injection; regenerative medicine

1. INTRODUCTION

Among musculoskeletal disorders, joint problems such as osteoarthritis (OA) rank first representing one of the major causes of lameness in canine and equine species, thus influencing the animal welfare and sportive performance in the case of racehorses [1,2]. OA is a chronic degenerative joint disease and represents a one-health challenge as it is one of the leading causes of disability in human patients worldwide. Furthermore, there is a close analogy between human and veterinary patients, making dogs and horses useful OA clinical models [3,4]. Despite the high prevalence in canine, equine, and even human populations, there is still a lack of successful long-term treatments that prevent or reverse osteoarthritis, improving the patient's quality of life without side effects [5]. Recently, biological therapeutics such as Mesenchymal Stromal Cells- based therapies, including cell-

free products such as conditioned medium and extracellular vesicles, have shown promising regenerative potential both in vitro and in vivo in pre-clinical studies [6-13]. However, to better understand the therapeutic activity of MSCs-based therapies, using naturally occurring diseases instead of model-based studies is closer to reality, especially in musculoskeletal disorders, characterized by a complex healing process. Furthermore, spontaneous disorders represent a suitable “one-health” model as the pathophysiology is very similar between canine, equine, and human species [14].

In recent years, various allogeneic cell-based veterinary products have become available in commerce, but data regarding animal clinical trials are still poorly represented in the literature [15]. As far as we know, only two research publications investigated the use of cell-free therapy using MSCs for the management of naturally occurring canine osteoarthritis, indicating the need for multicentric randomized controlled clinical trials in this field [16]. Regarding the equine species, at the time of writing, no studies on spontaneous OA have been conducted using MSCs-derived secretome or EVs, although in vitro and in vivo preclinical studies suggested that MSCs-derived cell-free therapies could reduce joint inflammation [7,10].

In the pilot study published in 2021, we assessed the safety of a double intra-articular injection of Lyo-secretome in client-owned dogs with bilateral elbow or knee osteoarthritis [17]. The present study represents the second part of the project, in which further batches of canine and equine batches of Lyo-secretome were prepared and characterized by following the GMP-compliant process described in the previous papers [17-19]. Since no published data are available regarding MSCs-derived cell-free products in equine naturally occurring osteoarthritis, 5 horses with spontaneous fetlock OA were treated with double injections of different doses of Lyo-secretome to assess preliminary safety data. Furthermore, 21 dogs were enrolled to evaluate both safety and efficacy. Clinical evaluations, orthopedic visits, and owner questionnaires have been used to monitor the patients during the 180-day follow-up.

2. MATERIALS AND METHODS

2.1 Materials

The Lyo-secretome production was prepared at an accredited facility to produce and control veterinary products for clinical use (Istituto Zooprofilattico Sperimentale Lombardia and Emilia-Romagna, IZLER, Brescia, Italy). The production protocol has been approved by the Italian Ministry

of Health (Prot. n. 0000778 del 15/01/2020 7.1.2.0.0.0/17/2019-AGD 809). Adipose-derived canine and equine MSCs were obtained by IZLER Biobank. All reagents for cell culture, e.g., DMEM, fetal bovine serum (FBS), and antibiotics, were purchased from Thermofisher Scientific, Milan, Italy. Mannitol was bought from Merck, Milan, Italy.

2.2 Preparation and characterization of Lyo-secretome batches

Canine and equine Lyo-secretome batches were prepared at an accredited facility to produce and control veterinary products for clinical use (Istituto Zooprofilattico Sperimentale Lombardia and Emilia-Romagna, IZLER, Brescia, Italy) following the production protocol approved by the Italian Ministry of Health (Prot. n. 0000778 del 15/01/2020 7.1.2.0.0.0/17/2019-AGD 809), and as previously described [17]. Briefly, adipose-derived MSCs (AD-MSCs) were seeded and expanded into T175 flasks at 10,000 cells/cm² density until P3 with DMEM added with 10% v/v FBS, plus 1% v/v penicillin/streptomycin and 1% v/v amphotericin B at 37 °C and 5% CO₂. The MSC-secretome release was induced by culturing MSCs in DMEM without fetal bovine serum (starvation) for 48 h; the supernatants were then collected, centrifuged at 3500× g for 10 min to remove cell debris, and ultrafiltered by tangential flow filtration (KrosFlo® Research 2i system, Spectrum Laboratories, Milan, Italy) using a filtration module with a Molecular Weight Cut Off (MWCO) of 5 kDa; during the process, the shear rate of the feed stream was maintained between 2000 s⁻¹ and 6000 s⁻¹, while trans-membrane pressure did not exceed 5 psi to avoid stresses that can rupture EV membrane. Once the concentration of 0.5×10⁶ cell equivalents per mL was reached, the ultrafiltration process was stopped, and the obtained sample was added with 0.5% w/v mannitol (as cryoprotectant) and freeze-dried (Christ Epsilon 2–16 D LSCplus) at -50 °C and 10-1 mbar for 72 h. The obtained Lyo-secretome batches were stored at -20 °C until use (48 months).

Lyo-secretome was obtained from MSCs that accomplished the requirements for clinical use in terms of cell viability, cellular identity, and genomic stability, as stated by the International Society for Cellular Therapy [20]. For each Lyo-secretome batch, total protein and lipids were dosed, the physical-chemical properties and the EV size and concentrations were determined according to the procedures reported and validated previously [19]; according to the provisions of the European Pharmacopoeia, further tests included the evaluation of sterility (EuPh 2.6.1), endotoxins using the Limulus Amebocyte Lysate (LAL) test (EuPh 2.6.14), and mycoplasma by NAT test (EuPh 2.6.7).

2.3 In vivo safety and efficacy evaluations

2.3.1. Ethical concerns

The clinical trial has been approved by the Italian Ministry of Health (Prot. n. 0000778 del 15/01/2020 7.1.2.0.0.0/17/2019-AGD 809). The ethical committee of Istituto Sperimentale della Lombardia ed Emilia Romagna (IZSLER, n. 1/2019, 21 March 2019) also approved the clinical experimentation. All patients were client-owned and all owners signed an informed consent before inclusion.

2.3.2. Study protocol

This was a double-blinded randomized controlled clinical trial enrolling animals suffering from naturally occurring osteoarthritis. All patients underwent complete blood and serum analysis and a clinical check to exclude concomitant systemic diseases. They also underwent orthopedic examinations and X-rays to confirm the starting grade of OA. Furthermore, patients should have suspended any kind of therapy, such as glucocorticoids or NSAIDs, before enrolment and for the entire period of the study, when compatible with the clinical state. No restrictions were placed in terms of sex, breed, or age, and only dogs weighing more than 15 kg were enrolled. The patients received a double intra-articular injection of Lyosecretome (or Mannitol for the control group), resuspended in Hyaluronic acid, at 40-day intervals. Clinical examinations were planned at different times. The study protocol is resumed in **Figure 1**.

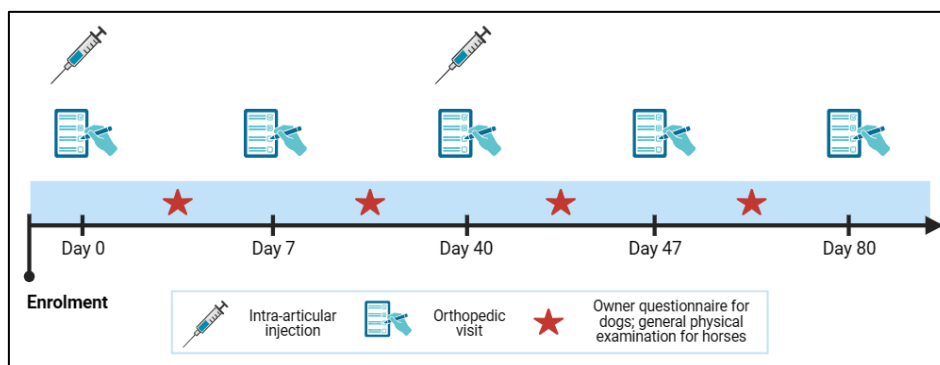


Figure 1. Workflow of the protocol. Intra-articular injection was planned on Day 0 and repeated after 40 days. An orthopedic visit was performed on Days 0,7,40,47 and 80. Owner questionnaires for dogs and general physical examinations for horses were planned at different times during the study protocol.

In detail, intra-articular injection was performed on Day 0 and Day 40. A complete orthopedic examination was assessed before each treatment and then repeated after 7 days. A final clinical

check was then planned on Day 80. Since for equine patients, a preliminary safety evaluation must be performed, a general physical examination was planned the next hours and days after each infiltration (2,4,8,12,24,36,72 hours after each injection, and on Day 20 and Day 60), while canine patients were monitored through a questionnaire containing the Helsinki Chronic Pain Index submitted to the owners 2,4,7, and 20 days after each treatment. Once the protocol was completed, an additional treatment with Lyosecretome resuspended in hyaluronic acid was offered to the patients treated with Mannitol.

2.3.4. Safety evaluation in equine patients

Five client-owned horses presenting naturally occurring fetlock osteoarthritis confirmed by radiographs and with clinical lameness persisting for at least 2 months were included in the study. Details of the clinical cases are resumed in **Table 1**.

Horse	Breed	Age (years)	Gender	Joint	Mono/bilateral
1	Italian trotter	26	F	Fetlock	Mono
2	Italian trotter	21	F	Fetlock	Mono
3	Italian trotter	16	F	Fetlock	Mono
4	Italian trotter	18	F	Fetlock	Mono
5	Italian trotter	16	F	Fetlock	Mono

Table 1. Summary of equine cases included in this study regarding breed, age, gender, and affected joint at the time of enrolling.

Pre-clinical and post-clinical evaluations included a static evaluation of pain and effusion of the affected joint using a numeric scale ranging from 0 to 3 (0=no pain/effusion, 1=mild pain/effusion, 2=moderate pain/effusion, 3=severe pain/effusion) and a dynamic evaluation of lameness through the flexion test and an adaptation of the AAEP (American Association of Equine Practitioners) grading system, on hard and soft ground, on a straight line and circle [21]. Furthermore, after each intra-articular injection, a local assessment of pain, heat, and effusion was performed, together with a general physical examination evaluating heart rate, respiratory rate, body temperature, CRT (capillary refill time), and appetite. The radiographic score, flexion test scale, and lameness grading system [21,22] are resumed in Supplementary Materials (File S1, Table 1-3). Finally, synovial fluid samples were collected in K3EDTA on Day 0, Day 40, and Day 80 to check cell count, total protein content, and cytologic analysis.

Animals were randomly assigned to different doses of Lyo-secretome, resuspended in a final volume of 3 mL of Hyaluronic Acid (2%, 100mg/5ml, Farmacia Piazza Bologna, Rome). The veterinarian and the owner were unaware of the joint's treatment. In detail, horse 1 represented the control patient and was treated with Mannitol, while horses 2,3,4, and 5 were treated with 0,5x10⁶, 0,8x10⁶, 1,2x10⁶, and 2x10⁶ cell equivalents respectively. Both Mannitol and Lyo-secretome were resuspended in hyaluronic acid. For each patient, the same dose was repeated on Day 40. Patients' treatments are resumed in **Table 2**.

Horse	Treatment/Dose (cell eq.)
1	Mannitol
2	0,5x10 ⁶
3	0,8x10 ⁶
4	1,2x10 ⁶
5	2x10 ⁶

Table 2. Patients' treatment with Mannitol or crescent doses of Lyo-secretome cell equivalents, resuspended in hyaluronic acid.

After examination, patients were sedated, and the skin over the joint was prepared for a sterile procedure. In detail, the 20G needle was introduced into the joint space, synovial fluid was aspirated and collected in K3EDTA tubes, and the treatment product was injected through the same needle and a syringe of 5 mL. Immediately after injection, a standard lower limb bandage was placed on the limb and maintained for 24 hours. After the procedure, the patient was hospitalized for 24 hours and then sent home, prescribing rest for the first three days. The local and general physical examinations were repeated in the next hours (2,4,8,12) and days (1,2,3, 7,20) after each treatment, while the orthopedic evaluation was performed 7 days after each injection.

2.3.5. Safety and Efficacy evaluation in canine patients

Twenty-one client-owned dogs weighing more than 15 kg and suffering from mono-lateral or bilateral elbow or knee osteoarthritis were enrolled (7 males and 14 females, mean age 8,31 ± 2,86; mean weight 30,47 ± 8,13). Details of clinical cases are shown in **Table 3**.

Dog	Breed	Age (years)	Gender	Weight (kg)	Joint	Mono/bilateral
1	German shepherd	7	F	30	Elbow	Mono
2	Mixed breed	13	F	16	Knee	Mono
3	Labrador	11	M	35	Elbow	Bi
4	Mixed breed	9	M	33	Knee	Mono
5	Mixed breed	11	F	30	Knee	Mono
6	Mixed breed	12	M	15	Knee	Bi
7	Labrador	10	M	39	Elbow	Mono
8	Mixed breed	5	M	22	Knee	Bi
9	Labrador	9	F	38,5	Elbow	Mono
10	Pittbull	10	F	32	Knee	Mono
11	Italian Bracco	1,5	M	30	Elbow	Mono
12	Mixed breed	9	F	28,5	Knee	Bi
13	Golden retriever	9	F	34	Elbow	Bi
14	Mixed breed	6	F	39,9	Elbow	Bi
15	Pointer	10	F	18	Elbow	Bi
16	Border collie	6	F	20	Knee	Mono
17	Labrador	8	F	35	Knee	Bi
18	Labrador	9	F	35	Elbow	Bi
19	Golden retriever	5	M	34	Elbow	Bi
20	Labrador	10	F	30	Elbow	Mono
21	Rottweiler	4	F	45	Elbow	Mono

Table 3. Summary of canine cases included in this study regarding breed, age, gender, weight, and affected joint at enrolment.

Since a preliminary safety evaluation of Lyo-secretome double intra-articular injection has already been conducted on canine clinical cases in the pilot paper of 2021, the present study aims to focus on the efficacy of these treatments in dogs, evaluating the same orthopedic parameters proposed previously [17]. Briefly, OA starting grade was assessed with X-rays, while a pre- and post-clinical orthopedic visit was performed including lameness, pain during palpation, functional disability, and range of motion evaluations [23,24]. The clinical and orthopedic scale systems used for this study are resumed in Supplementary Materials (**File S2, Table 1-4**). Furthermore, the measurement of the

range of motion was assessed as shown in **Figure 2**. In detail, while the orthopedic surgeon performed manual maximal joint flexion (**Figure 2, 1**) or extension (**Figure 2, 2**), specific landmarks were identified and a photo was taken on the mobile camera by an external assistant, keeping the camera in a perpendicular plan. The image was opened on the Angulus android app designed to measure angles (Angulus version 4.0). The angles subtended by the lines connecting the landmarks were immediately displayed on the screen of the smartphone application. This method was each time compared to the goniometric standard measure.

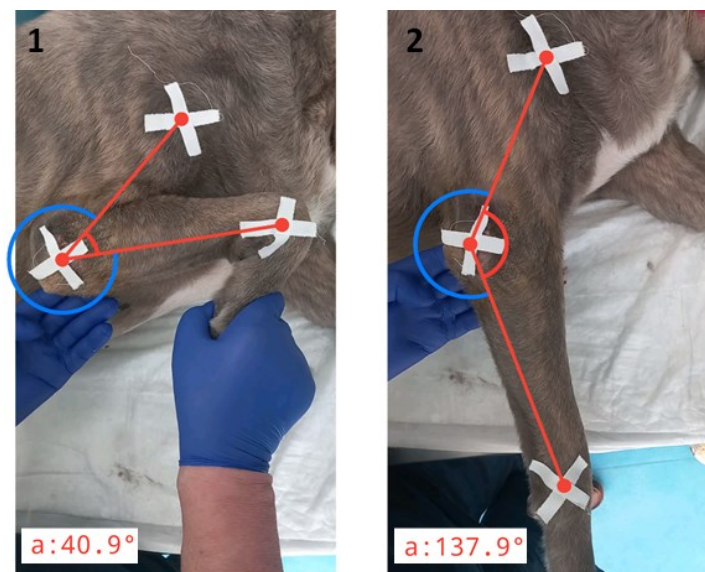


Figure 2. Steps of app goniometry—a picture was taken on the mobile camera by an external assistant, while the surgeon performed manual maximal flexion (1) or extension (2) of the joint. Landmarks were marked, and the angle between them was calculated automatically by the Angulus android app (1a: 40.9°; 2a: 137.9°).

Animals were randomly assigned to two different groups and treated with Lyo-secretome (20 mg corresponding to 2×10^6 cell equivalents, treatment group) or Mannitol (control group), both resuspended in 1 ml hyaluronic acid (Athenavis 1%, Ibn Savio, Pomezia, Italy), containing 20 mg of sodium hyaluronate (800–1300 KDa, in 2 mL). Research Randomizer was used to create the randomization. When bilateral, one joint was infiltrated with the Lyosecretome and the contralateral one with Mannitol. The intra-articular injection was performed as previously described [17]. Dogs were discharged from the clinic the same day, and recommendations were provided to the owners to restrict exercise for 2 days following injection and resume regular activity from the third day. During the follow-up, the orthopedic examination was repeated 7 and 40 days after each intra-articular treatment; furthermore, a questionnaire containing the validated Helsinki Chronic Pain Index [25-27] was submitted to the owners at different time points (2,4,7,20,40 days after each

injection), to check information about the dog's general conditions, the presence or absence of any adverse response, as well as the assessment of chronic pain sign (see **Supplementary Materials, File S2, Table 5**).

2.4 Statistical analysis

All analyses were performed using the statistical program R Core Team (2023) (R: A Language and Environment for Statistical Computing_. R Foundation for Statistical Computing, Vienna, Austria; <https://www.r-project.org/>) [28]. The lme4 package was used to analyze canine clinical outcomes [29].

3. RESULTS AND DISCUSSION

In two previous works, our group already validated the GMP-compliant production process to obtain Lyo-secretome from canine and equine species, also demonstrating the absence of cytotoxicity through in vitro potency tests, such as anti-elastase activity and proliferative activity on targeted cell types [17,18]. In this study, further canine and equine lyo-secretome batches have been prepared following the same GMP procedures in a facility accredited for the production of veterinary medicinal products [17,30,31] and characterized to assess their suitability for clinical application following intraarticular injection. In this regard, all the batches produced fulfilled the provision of the European Pharmacopoeia in terms of sterility (EuPh 2.6.1), absence of endotoxin (EuPh 2.6.14), and mycoplasma contamination (EuPh 2.6.7).

A molecular cut-off of 5 kDa was intentionally selected during the manufacturing step to retain both soluble proteins and EVs, as superior immune-modulatory and regenerative properties of the whole secretome were observed concerning only EV fraction [30,32]. **Table 4** reports the protein and lipid content for each batch prepared.

Batch	Animal specie	µg of proteins/mg lyo-secretome	µg of lipids/mg lyo-secretome
1	Canine (204)	24.2 ± 0.67	31.7 ± 1.03
2	Canine (220)	14.0 ± 0.083	2.65 ± 0.225
3	Equine (176)	9.3 ± 0.66	13.1 ± 3.80
4	Equine (201)	15.3 ± 0.54	10.2 ± 0.26

Table 4. Summary of the canine and equine lyo-secretome batches prepared and their characterization in terms of µg of proteins or lipids per mg of freeze-dried product. Data are reported as mean values standard deviation, n=3 measurements for each sample.

A batch-to-batch variability in the protein and lipid content is detectable within the same species despite the standardization of the culture conditions, even in terms of cell density. For example, the canine batch 1 contained more proteins and lipids concerning batch 2 from the same species; likely, equine batch 4 contained more proteins but fewer lipids than batch 3 from the same species. In this regard, it has to be considered that planar culture conditions are well known to be highly dependent on individual expertise, leading to batch-to-batch variability and reduced reproducibility [33-35]; furthermore, the ultrafiltration process is set to standardize the concentration of each batch in terms of cell equivalents (i.e., the number of cells that produced an exact amount of secretome) and not as protein/lipid content. As a result, the same number of cells may produce different amounts of secretome and thus proteins and lipids; in this regard, Mocchi and colleagues recently suggested that for more consistency among the batches produced, the product resulting from the ultrafiltration step should be standardized to a specific protein (and lipid) concentration [31].

The simultaneous presence of proteins and lipids was also assessed by physical-chemical characterization, i.e., Fourier Transform Infrared Spectroscopy, which revealed typical intensity bands of amide I C=O stretching vibrations and amide II N-H bending vibrations for proteins and the stretching vibrations typical of lipids (data not shown). For all batches, the thermogravimetric analysis revealed a mass loss lower than 2.5% in the temperature range of 50-80 °C (due to dehydration), suggesting that the freeze-dried process was conducted successfully (curve not reported). NTA characterization detected a heterogeneous EV population in all batches (**Table 5** and **Figure 3**), consistent with retaining the whole secretome, even including the entire EV population and not only the small EVs (exosomes). In detail, the d10 for all batches ranged from 87-101 nm, the d50 ranged from 127-148 nm, and the d90 was less than 350 nm.

Batch	Mean	Mode	d ₁₀	d ₅₀	d ₉₀
Canine (204)	171.8 ± 1.9	109.9 ± 5.2	101.0 ± 1.7	148.2 ± 2.0	265.4 ± 7.4
Canine (220)	150.5 ± 3.2	94.3 ± 3.4	87.2 ± 1.0	127.5 ± 3.3	234.4 ± 11.4
Equine (176)	171.7 ± 4.0	102.5 ± 3.2	87.3 ± 2.1	128.7 ± 3.3	288.1 ± 12.2
Equine (201)	184.9 ± 7.2	96.9 ± 3.5	95.3 ± 3.0	144.7 ± 6.0	348.9 ± 19.8

Table 5. EV particle size for each lyo-secretome batch prepared. Data are reported as mean values standard deviation (n=6 measurements for each sample) and expressed in nm.

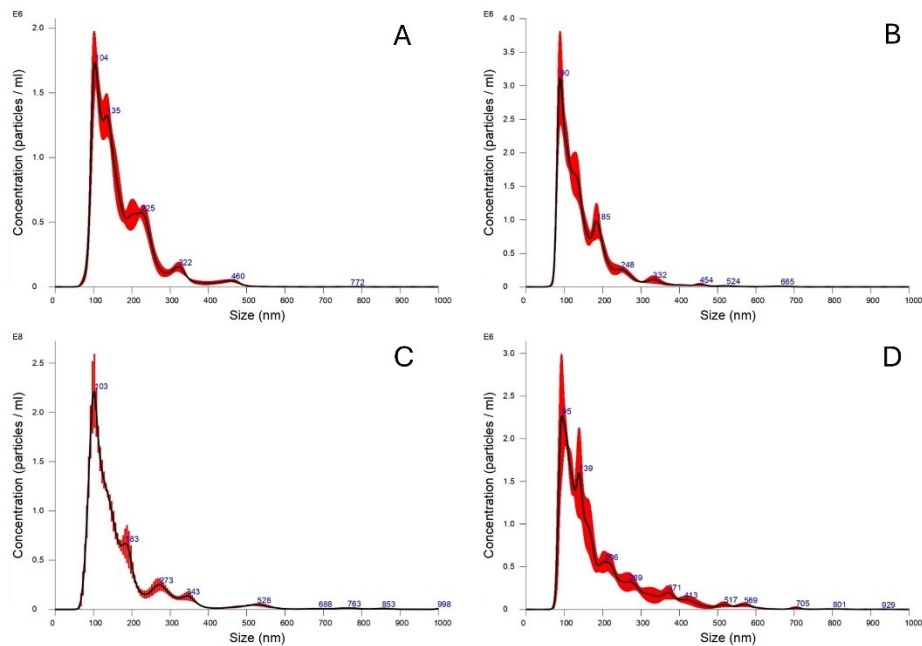


Figure 3. EV particle size and concentration for batches 1 (A), 2 (B), 3 (C), and 4 (D). The averaged curve was generated by processing six cycles of 60 s each per batch. Concentration is indicated as mean value standard deviation (n = 3). The red part shows the standard error of the mean values (n = 3).

The emerging field of cell-free therapies represents a promising approach to treating joint disorders in dogs and horses. Regarding the equine species, based on the results presented in some in vitro and in vivo pre-clinical studies, MSCs-derived secretome, and EVs seem to reduce joint inflammation [7,10], although no in vivo studies on spontaneous OA have been conducted in horses. The present study enrolled five horses spontaneously suffering from fetlock osteoarthritis and treated with a double intra-articular injection of Lyo-secretome to collect preliminary safety data. Lyo-secretome was prepared at different final concentrations and tested in a dose ranging from 0,5 to 2×10^6 cell equivalents and resuspended in hyaluronic acid, to ensure both an effective therapy and a lubrication activity for the affected joints. No systemic adverse responses were observed in any patients, even after the second intra-articular injection of the allogeneic product. During the general clinical examinations, all the parameters recorded were indeed within the physiological range at all the different time points. Regarding the local joint examination, all the patients presented mild to moderate swelling in the treated joint, including the control horse. In detail, Horse 2 presented a slight inflammation with joint swelling and periarticular heat restored in 12 hours, while Horse 1 and 4 developed the same transient response for 24 hours post-injection. Horses 3 and 5 presented a moderate local response for up to 48 hours, but in Horse 5 the swelling was extra-articular and proximal to the fetlock and has been correlated with the application of the bandage, as the joint

distension was the same compared to time 0. A similar trend was observed for the second injection, with no significant differences for all the patients. All the reactions were both times self-limiting and resolved with no anti-inflammatory medication. The presence of a mild adverse response even in Horse 1, treated with Mannitol resuspended in HA, suggested that these local reactions were not directly related to the administration of Lyo-secretome, as they appear even when administering the placebo. Details of the local and general physical examinations are reported in **Supplementary Materials, File S3**. The reported effects are in line with the published literature, as frequently, no differences can be found between the treated group and the control group undergoing the same injection protocol suggesting a possible non-specific inflammation related to the arthrocentesis procedure itself or the severity state of OA joints [22]. Furthermore, a self-limiting joint flare is commonly noticed in horses treated with intra-articular administration of different injectable products [22,36-38]. Interestingly, in our study, the local response seemed not correlated with a dose-dependent effect, since a similar trend was observed independently of the treatment. The synovial fluid analysis did not show any significant changes in terms of cell count, total protein, and cytology pre- and post-treatment in all the treated horses. Finally, regarding the clinical data, the orthopedic examination performed by the veterinarian 7 and 40 days after each injection was used to assess the possible onset of unexpected symptoms after the treatment. No evidence of lameness or pain worsening was noticed. On the contrary, an improvement was observed in terms of lameness and pain for these parameters in all the treated animals, but the limited number of enrolled patients does not provide any direct evidence of the efficacy of this treatment, and the clinical results will be discussed when reaching a proper number of cases.

Regarding the canine species, twenty-one dogs (31 joints) with monolateral or bilateral elbow or knee OA were randomly assigned to the treatment group or the control group. A total of 14 joints were treated with HA, while 17 were treated with Lyo-secretome. Each patient received a set of two injections of the same product at a 40-day interval. No systemic adverse responses were observed in any patient. Regarding the local effects, based on the owner's survey, eight patients out of 21 (38,1%) presented a self-limiting joint flare the next day after the injection, with apparently no differences between treatments since a total of 4 joints have been treated with HA and 6 joints with Lyo-secretome. In detail, two patients with bilateral OA (Dog 3 and Dog 18) presented moderate inflammation in both joints, despite the different treatment received; a mild reaction occurred after the second injection only in Dog 3. In Dogs 5 (treated with HA), and in Dogs 6 and 7 (treated with

Lyo-secretome), a mild local response was observed after both treatment points with no worsening. Dog 10 presented a very mild joint flare after the first injection of Lyo-secretome, while no adverse reactions were noticed after the second treatment. Finally, Dogs 4 and 8 presented a similar moderate inflammation after the second injection of respectively HA and Lyo-secretome, once again suggesting the absence of a treatment-dependent effect. Notably, 7 of 8 of these patients presented a severe OA starting grade ranging from 4 to 5, except for Dog 10 who presented grade 3 OA, suggesting that the post-treatment response could be correlated to the severity of the OA state of the joint. All the local reactions were restored in 24 (Dogs 3,4,6,7,8,10) or 48 hours (Dogs 5 and 18); analgesic administration was accepted only the first day after the injection for Dogs 5, 6, 7, and 18 as requested by the owner, while the other local responses spontaneously restored with no side effects. The reported effects are usually mild and self-limiting and commonly found in other published clinical studies involving both MSCs-based products and conventional treatments such as HA [23,39-43]. Overall, no statistically significant differences were found between the two groups in terms of owner evaluation of pain or swelling of the treated joint (**Supplementary materials, File S4**). Clinical evaluations assessing lameness, pain during palpation, range of motion, and functional disability were used as clinical outcome measures during the 180-day study period. All the patients completed the follow-up appointments. Hyaluronic Acid was selected as a control group as it is readily available to veterinary clinicians and reported to have beneficial effects on naturally occurring OA [41,44]. Furthermore, comparison with conventional treatment is more clinically relevant than choosing no treatment as a control group. We expected to see a clinical improvement in the HA group, but not better than those observed for the Lyo-secretome group. Our preliminary data are in line with this hypothesis since both groups showed evidence of an improvement following the treatment; however, despite the presence of a positive tendency for lameness and pain evaluation in the Lyo-secretome group, the small sample size does not allow a statistically significant difference between the two treatments. No differences were found also for the range of motion and the functional disability.

Regarding the owner surveys, bilateral OA patients were excluded as they received both treatments and the questionnaire didn't allow to separate the effects of the two different products. For monolateral OA patients, both HA and Lyo-secretome applications showed an improvement assessed by the 11 questions of the HCPI (**Figure 4**), cited in the literature as clearly able to

discriminate between healthy and painful dogs and to detect pain severity decrease after pain treatment [45,46].

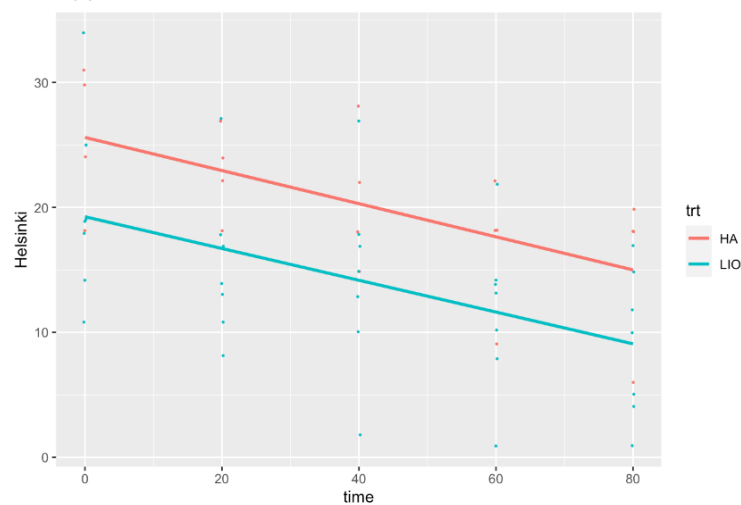


Figure 4. Helsinki Chronic Pain Index (HCPI) scores before treatment (time 0) and over time in dogs treated with intra-articular hyaluronic acid (HA, red line) or Lyo-secretome (LIO, blue line).

Interestingly, a tendency for a better improvement was found for the question “dog’s willingness to walk” after Lyo-secretome treatment, even if not statistically significant ($p= 0.0714$). Since healthy dogs have an HCPI between 0 and 6, or even values up to 11 assuming that an individual score of 1 is considered normal [45], once again the outcome improvement of the Lyo-secretome group appeared to be more consistent, even if not significant.

4. CONCLUSIONS

The present study confirmed the proof-of-concept for veterinary clinical-grade MSC-secretome production for both canine and equine species; moreover, the feasibility of two consecutive allogeneic Lyosecretome intraarticular administration in osteoarthritic patients is proved, without evidence of systemic adverse reactions. Although mild to moderate joint flare was observed in both species, the local reactions were self-limiting and in line with the results presented in other clinical studies on in-tra-articular injections in animals suffering from spontaneous OA, with no differences from the HA group. To our knowledge, this is the first study evaluating a cell-free product derived from MSCs on horses with naturally occurring OA. Regarding the efficacy in canine patients, our study revealed mild improvement following the two treatment injections in both groups, without highlighting one treatment to be superior. This could be due to different reasons. First of all, the number of enrolled patients is a clear limit of the study and does not provide any direct evidence of

the efficacy or safety of the Lyosecretome in both species. Besides the sample size, cell-free therapies are still poorly represented in veterinary clinical trials, and no standardized protocols are available, especially in terms of doses and administration frequency. A huge variability existed also in terms of the study population, since both elbow and knee joints, as well as single or multiple OA joints, were included in the study. Further research into the safety and efficacy of this treatment must be pursued to draw firm conclusions.

5. SUPPLEMENTARY MATERIALS

File S1

EQUINE CLINICAL SCORE SYSTEMS

Parameter	Score	Definition
Pain	0	Absent
	1	Mild
	2	Moderate
	3	Severe
Joint effusion	0	Absent
	1	Mild
	2	Moderate
	3	Severe
Flexion test	0	No lameness increase
	1	Slight lameness increase
	2	Moderate lameness increase
	3	Marked lameness increase
	4	Severe lameness increase

Table 1. Overview of the score systems used for the orthopaedic examination.

Parameter	Score	Definition
A: Subchondral bone /epiphyseal bone changes	0	No significant changes
	1	Diffuse sclerosis, subchondral bone thickening
	2	Focal or marginal subchondral bone lysis, cyst-like subchondral lesions
	3	Diffuse or multifocal subchondral bone lysis
B. Articular space changes	0	No significant changes
	1	Mild space collapse
	2	Moderate space collapse
	3	Severe space collapse
	4	Ankylosis
C. Periarticular new bone formation (osteophytes and enthesophytes)	0	No significant changes
	1	Mild remodeling of articular margins
	2	Moderate remodeling with spur-like formations
	3	Severe to exuberant new bone formation
Total OA score=	A+B+C	

Table 2. The OA grading system for fetlock joint in horses.

Lameness score	Description
0	Lameness not perceptible under any circumstances
1	Lameness is difficult to observe and is not consistently apparent, regardless of circumstances (e.g. under saddle, circling, inclines, hard surface, etc.)
2	Lameness is difficult to observe at a walk or when trotting in a straight line but consistently apparent under certain circumstances (e.g. weight-carrying, circling, inclines, hard surface, etc.)
3	Lameness is consistently observable at a trot under all circumstances
4	Lameness is obvious at a walk
5	Lameness produces minimal weight bearing in motion and/or at rest or a complete inability to move

Table 3. The lameness scale system by AAEP guidelines.

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CANINE CLINICAL SCORE SYSTEMS

Table 1. Overview of the score systems used for the canine orthopedic examination.

Parameter	Score	Definition
Lameness	1	Stands, walks and trots normally
	2	Stands normally, slightly painful gait when trotting
	3	Stands normally, slightly painful gait when walking
	4	Stands normally, evident painful gait when walking
	5	Stands abnormally, evident painful gait when walking
Pain	1	None
	2	Mild signs
	3	Moderate signs
	4	Severe signs
	5	The dog will not allow palpation
Functional disability	1	Normal
	2	Slightly stiff
	3	Stiff
	4	Very stiff, unwilling to walk
	5	Need assistance to walk
Range of motion	1	No limitation of movement or crepitus
	2	10 to 20% decrease in range of motion, no crepitus
	3	10 to 20% decrease in range of motion with crepitus
	4	20 to 50% decrease in range of motion
	5	More than 50% decrease in range of motion

Table 2. The Kellgren-Lawrence grading of osteoarthritis for the knee joint.

OA	Grading	Radiographic features
Absent	0	No abnormalities
Doubtful	1	Minute osteophytes
Minimal	2	Definite osteophytes
Moderate	3	Diminished joint space
Severe	4	Greatly diminished joint space + sclerosis of the subchondral bone

Table 3. International Elbow Working Group (IEWG) guidelines for elbow osteoarthritis.

OA	Grading	Radiographic features
Absent	0	Normal elbow joint, no evidence of incongruency, sclerosis, or arthrosis
Mild	1	Presence of osteophytes < 2 mm high, sclerosis of the base of the coronoid process—trabecular pattern still visible
Moderate	2	Presence of osteophytes of 2-5 mm high, obvious sclerosis (no trabecular pattern) of the base of the coronoid processes
Severe	3	Osteophytes of over 5 mm found anywhere in the joint

Table 4. Disease score obtained by adding the scores of the parameters evaluated in this study.

Sum of scores	Disease score
0 - 2	1
3 - 6	2
7 - 10	3
11 - 14	4
15 - 20	5

Question asked	0 points	1 point	2 points	3 points	4 points
D2. Pain upon right joint palpation	Absent	Mild	Moderate	Severe	Does not allow palpation
D3. Pain upon left joint palpation	Absent	Mild	Moderate	Severe	Does not allow palpation
D4. Swelling upon right joint palpation	Absent	Mild	Moderate	Severe	Does not allow palpation
D5. Swelling upon left joint palpation	Absent	Mild	Moderate	Severe	Does not allow palpation
D6.1 Heat in the right joint	No warmer than contralateral limb	Mildly warmer	Moderately warmer	Markedly warmer	
D6.2 Heat in the left joint	No warmer than contralateral limb	Mildly warmer	Moderately warmer	Markedly warmer	
D7. Rate your dog's mood	Very alert	Alert	Neither alert nor indifferent	Indifferent	Very indifferent
D8. Rate your dog's willingness to participate in play	Very willing	Willing	Reluctantly	Very reluctantly	Does not play at all
D9. Rate your dog's complaining and groaning	Never	Hardly ever	Sometimes	Often	Very often
D10. Rate your dog's willingness to walk	Very willing	Willing	Reluctantly	Very reluctantly	Does not walk at all
D11. Rate your dog's willingness to trot	Very willing	Willing	Reluctantly	Very reluctantly	Does not trot at all
D12. Rate your dog's willingness to gallop	Very willing	Willing	Reluctantly	Very reluctantly	Does not gallop at all
D13. Rate your dog's willingness to jump (e.g., into a car, onto a sofa)	Very willing	Willing	Reluctantly	Very reluctantly	Does not jump at all
D14. Rate your dog's ease in lying down	With great ease	Easily	Neither easily nor difficultly	With difficulty	With great difficulty
D15. Rate your dog's ease in rising from a lying position	With great ease	Easily	Neither easily nor difficultly	With difficulty	With great difficulty
D16. Rate your dog's ease of movement after long rest	With great ease	Easily	Neither easily nor difficultly	With difficulty	Very often/always difficulty
D17. Rate your dog's ease of movement after major activity or heavy exercise	With great ease	Easily	Neither easily nor difficultly	With difficulty	Very often/always difficulty
D18. Dog's appetite	Normal	Capricious	Decreased	Markedly decreased	Does not eat at all
D19. Dog's body temperature	Normal	Fever			
D20. Use of analgesic/anti-inflammatory drugs	No	Yes			
D21. Abnormal post-treatment reactions	None	Physical tiredness	Physical tiredness + joint pain/swelling	Increased lameness	Does not stand and walk at all

Table 5. Owner questionnaire containing the Helsinki Chronic Pain Index and some questions regarding presence or absence of local/systemic adverse responses.

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EQUINE GENERAL AND LOCAL EXAMINATIONS

Since no statistically significant differences were observed between the first and the second injection, the reported parameters refer to the first treatment.

Case 1: Italian Trotter, 26 years, female, treated with Mannitol resuspended in Hyaluronic Acid.

Time	Body temperature (°C)	Heart rate (Beats per min)	Respiratory rate (Breaths per min)	CFT (capillary filling time)	Appetite	Joint swelling	Joint heat	Pain during palpation
0	36,9	40	16	< 2	normal	no	no	no
2h	37,1	42	12	< 2	normal	no	no	no
4h	36,8	40	12	< 2	normal	no	no	no
6h	36,9	36	12	< 2	normal	mild	mild	no
8h	37,1	40	16	< 2	normal	mild	mild	no
12h	37,5	36	18	< 2	normal	mild	mild	no
24h	36,9	32	12	< 2	normal	mild	mild	no
48h	36,9	32	16	< 2	normal	no	no	no
72h	37,5	40	16	< 2	normal	no	no	no
7 days	37,1	40	12	< 2	normal	no	no	no

Case 2: Italian Trotter, 21 years, female, treated with $0,5 \times 10^6$ cell equivalents of Lyo-secretome resuspended in Hyaluronic Acid.

Time	Body temperature (°C)	Heart rate (Beats per min)	Respiratory rate (Breaths per min)	CFT (capillary filling time)	Appetite	Joint swelling	Joint heat	Pain during palpation
0	37,6	32	12	< 2	normal	no	no	no
2h	36,9	36	12	< 2	normal	no	no	no
4h	37,1	32	12	< 2	normal	no	no	no
6h	37,3	32	12	< 2	normal	Mild	Mild	no
8h	37,1	32	18	< 2	normal	mild	mild	no
12h	37,1	28	16	< 2	normal	mild	mild	no
24h	36,9	28	12	< 2	normal	no	no	no
48h	36,9	32	16	< 2	normal	no	no	no
72h	37,5	32	16	< 2	normal	no	no	no
7 days	37,2	32	12	< 2	normal	no	no	no

Case 3: Italian Trotter, 16 years, female, treated with $0,8 \times 10^6$ cell equivalents of Lyo-secretome resuspended in Hyaluronic Acid.

Time	Body temperature (°C)	Heart rate (Beats per min)	Respiratory rate (Breaths per min)	CFT (capillary filling time)	Appetite	Joint swelling	Joint heat	Pain during palpation
0	37,5	48	12	< 2	normal	No	No	No
2h	37,3	36	12	< 2	normal	Mild	Mild	No
4h	37,1	32	12	< 2	normal	Mild	Mild	No
6h	37,3	32	18	< 2	normal	Moderate	Mild	No
8h	37,1	32	12	< 2	normal	Moderate	Mild	No
12h	37,8	32	12	< 2	normal	Moderate	Mild	No
24h	37,5	32	16	< 2	normal	Moderate	No	No
48h	37,3	40	12	< 2	normal	Moderate	No	No
72h	37,8	36	18	< 2	normal	No	No	No
7 days	37,5	36	16	< 2	normal	No	No	No

Case 4: Italian Trotter, 18 years, female, treated with $1,2 \times 10^6$ cell equivalents of Lyo-secretome resuspended in Hyaluronic Acid.

Time	Body temperature (°C)	Heart rate (Beats per min)	Respiratory rate (Breaths per min)	CFT (capillary filling time)	Appetite	Joint swelling	Joint heat	Pain during palpation
0	36,8	40	12	< 2	normal	no	no	no
2h	37,2	42	12	< 2	normal	no	no	no
4h	36,9	40	16	< 2	normal	no	no	no
6h	36,9	32	12	< 2	normal	mild	mild	no
8h	37,2	32	16	< 2	normal	mild	mild	no
12h	37,2	36	12	< 2	normal	mild	mild	no
24h	36,9	40	12	< 2	normal	mild	mild	no
48h	36,9	32	12	< 2	normal	no	no	no
72h	37,5	40	16	< 2	normal	no	no	no
7 days	37,2	40	12	< 2	normal	no	no	no

Case 5: Italian Trotter, 16 years, female, treated with 2×10^6 cell equivalents of Lyo-secretome resuspended in Hyaluronic Acid. The moderate swelling reported by the veterinarian after the first intra-articular injection was diagnosed as extra-articular due to the presence of the bandage. Only a mild joint flare was observed, restored in 24 hours.

Time	Body temperature (°C)	Heart rate (Beats per min)	Respiratory rate (Breaths per min)	CFT (capillary filling time)	Appetite	Joint swelling	Joint heat	Pain during palpation
0	37,7	36	12	< 2	normal	no	no	no
2h	37,8	40	16	< 2	normal	no	no	no
4h	37,5	38	16	< 2	normal	no	no	no
6h	37,5	36	12	< 2	normal	mild	mild	no
8h	37,5	36	16	< 2	normal	mild	mild	no
12h	37,7	38	18	< 2	normal	mild	mild	no
24h	37,6	38	16	< 2	normal	mild	mild	no
48h	37,8	36	12	< 2	normal	no	no	no
72h	37,4	38	16	< 2	normal	no	no	no
7 days	37,5	38	16	< 2	normal	no	no	no

OWNER EVALUATION OF PAIN AND SWELLING

Question D2-D3: Pain upon joint palpation

	Estimate	p.z
(Intercept)	4.197172e-01	0.005093362
time	7.505116e-05	0.960194632
trtLIO	4.255089e-03	0.983222498
time:trtLIO	-3.863358e-03	0.057101042

[1] "At time 0"

Welch Two Sample t-test

data: question by trt
t = 1.5426, df = 306.35, p-value = 0.1239
alternative hypothesis: true difference in means between group HA and group LIO
is not equal to 0
95 percent confidence interval:
-0.03231372 0.26684389
sample estimates:
mean in group HA mean in group LIO
0.4220779 0.3048128

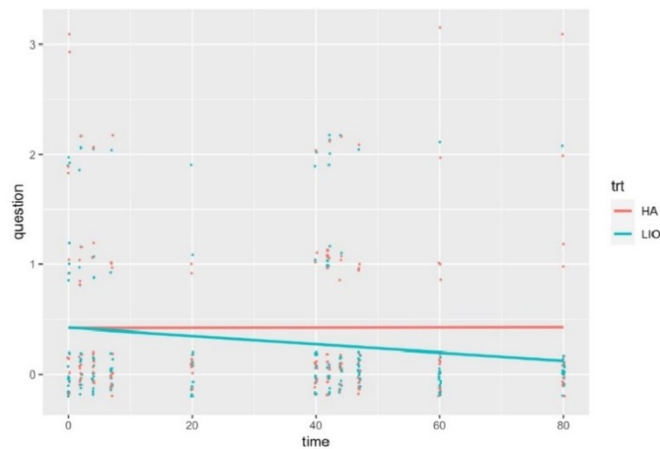


Figure 1. Owners' answer to the question "Pain upon joint palpation" (D2-D3) in mono-lateral patients at different time points (0,2,4,7,20,40 days after each injection). No differences were found between the treatment (LYO) and the control group (HA).

Question D4-D5: Swelling upon joint palpation

```

              Estimate      p.z
(Intercept)  0.142972465 0.06086804
time         -0.001861635 0.12377854
trtLIO       0.227882142 0.02693469
time:trtLIO  -0.001598041 0.32788895
`geom_smooth()` using formula = 'y ~ x'

```

[1] "At time 0"

Welch Two Sample t-test

```

data: question by trt
t = -3.8855, df = 303.96, p-value = 0.0001254
alternative hypothesis: true difference in means between group HA and group LIO
is not equal to 0
95 percent confidence interval:
 -0.26757058 -0.08766242
sample estimates:
mean in group HA mean in group LIO
  0.08441558      0.26203209

```

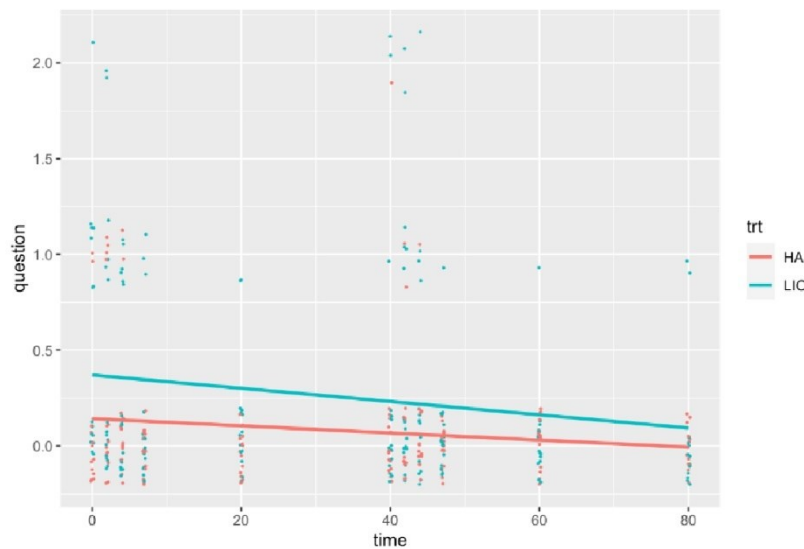


Figure 2. Owners' answer to the question "Swelling upon joint palpation" (D3-D4) in mono-lateral patients at different time points (0,2,4,7,20,40 days after each injection). No differences were found between the treatment (LYO) and the control group (HA).

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General results and conclusions

The management of osteoarthritis represents a big challenge for veterinary practitioners. OA indeed requires a proper diagnosis and a multimodal approach to treat both the primary site inflammation and the secondary effects on soft tissues. Despite OA causes significant morbidity in dogs, horses, and even humans worldwide, there is still a lack of successful therapy for the long-term treatment and prevention of OA. Owners are also seeking alternative treatment options to surgery or conservative management and therefore regenerative medicine is becoming a more popular treatment approach. Between the different therapeutic strategies, biological options such as platelet concentrates or Mesenchymal Stromal Cells-derived products have become more common in recent years, due to the advancement of research regarding their promising regenerative potential both in vitro and in vivo in pre-clinical studies [32,33,35,36,83,84,86]. Furthermore, these therapies could have a disease-modifying effect associated with a positive impact on patients' quality of life. Currently, the veterinary interest in using cell therapies is progressing, stimulating the development and research on different cell-based products with a focus on musculoskeletal disorders (MSDs). EMA (European Medicines Agency) indeed already approved the marketing authorization of some equine and canine allogeneic and xenogeneic MSCs-based products for the treatment of MSDs, as a response to an increasing demand in the veterinary medical practice [9]. It is currently known that the therapeutic effects of MSCs are mainly due to the release of their secretome, composed of extracellular vesicles and soluble factors such as cytokines, chemokines, and growth factors. Among regenerative products, the use of cell-free therapies as MSCs-secreted factors and vehicles instead of cells themselves is gaining popularity in the scientific world as they represent a new option to enhance the standardization, affordability, safety, and efficacy of this approach [51]. Being MSCs active and viable cells, they are very sensitive to different external conditions such as temperature, time, pH, and suspension vehicle; conversely, their derived cell-free products offer many practical advantages since they are easy to store for the long-term and ready-to-use like a conventional drug.

In this scenario, Bari and colleagues presented a GMP-compliant human secretome production process to obtain a freeze-dried form of secretome, called Lyo-secretome, from human adipose tissue-derived MSCs [10–14 m]. The process included an ultrafiltration step to concentrate and purificate MSCs-derived secretome, and the following lyophilization step to obtain a final powder with long-term stability [30,31].

The present thesis work described the preparation and characterization of the Lyo-secretome from adipose tissue-derived mesenchymal stromal cells in canine and equine species. The final batches were then characterized in terms of protein and lipid content, EV size distribution and tested to ensure microbiological safety. For equine batches, a proteomic analysis determined the presence of proteins involved in the regulation of the inflammatory process. The proof-of-concept for veterinary clinical-grade MSC-secretome production for both canine and equine species was confirmed. The second step was the assessment of in vitro biological effects of Lyo-secretome through potency tests, such as anti-elastase activity and proliferative activity, on targeted cell types such as primary culture of tendons and cartilage, and synovial fluid derived-MSCs. The results confirmed the absence of cytotoxicity and the presence of proliferative effects for both canine and equine cell populations. The next step was the production of canine and equine Lyo-secretome batches, validated to satisfy all the safety and sterility requirements needed for injectable pharmaceutical products as the final aim was to test the in vivo safety and efficacy of this product for the treatment of joint diseases. In general, to better understand the therapeutic activity of MSCs-based therapies, using naturally occurring diseases instead of model-based studies is closer to reality, especially in musculoskeletal disorders, characterized by a complex healing process. Furthermore, spontaneous disorders represent a suitable “one-health” model as the pathophysiology is very similar between canine, equine, and human species [46].

To this aim, a double-blinded randomized controlled clinical trial was performed collecting preliminary safety and efficacy data of a double intra-articular injection of Lyo-secretome resuspended in Hyaluronic acid, in dogs and horses with naturally occurring osteoarthritis. In detail, five horses with spontaneous fetlock OA were treated with double injections of different doses of Lyo-secretome to assess preliminary safety data. Regarding the canine species, 21 dogs were enrolled to evaluate both safety and efficacy. Clinical evaluations, orthopedic visits, and owner questionnaires have been used to monitor the patients during the 180-day follow-up.

The feasibility of two consecutive allogeneic Lyosecretome intraarticular administration in osteoarthritic patients has been proved, without evidence of systemic adverse reactions. Although mild to moderate joint flare was observed in both species, the local reactions were self-limiting and in line with the results presented in other clinical studies on intra-articular injections in animals suffering from spontaneous OA, with no differences from the HA group. To our knowledge, this is the first study evaluating a cell-free product derived from MSCs on horses with naturally occurring

OA, and only one published paper investigated the use of MSCs-derived conditioned medium for the management of naturally occurring canine osteoarthritis, indicating the need for multicentric randomized controlled clinical trials in this field [51]. Regarding the efficacy in canine patients, our study revealed mild improvement following the two treatment injections in both groups, without highlighting one treatment to be superior.

Overall further studies are needed to define well-characterized protocols for the use of MSCs-derived cell-free therapies, starting from the production methods to the characterization and final composition, defining the type and stage of disease to be treated, as well as the dosage, route of administration, and so on. The present thesis represents one of the first efforts to bring new regenerative treatments into the outpatient clinical setting in naturally occurring veterinary diseases, thus providing valuable information for the human counterpart, in a One Health-One Medicine approach.

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