Sildenafil improves clinical signs and radiographic features in dogs with congenital idiopathic megaoesophagus: a randomised controlled trial.

SILDENAFIL IMPROVES CLINICAL SIGNS AND RADIOGRAPHIC FEATURES IN DOGS WITH CONGENITAL IDIOPATHIC MEGAOESOPHAGUS: A RANDOMISED CONTROLLED TRIAL

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Abstract

We evaluated the efficacy of oral sildenafil citrate in dogs with congenital idiopathic megaoesophagus (CIM). Twenty-one puppies were randomly assigned to two groups (treatment and control). The dogs were given sildenafil oral suspension 1 mg/kg every 12 hours for 14 days or placebo in a masked fashion. Clinical signs (frequency of regurgitation and weight gain) and oesophagrams (relative oesophageal diameter, ROD) were evaluated in order to assess the efficacy of drug treatment, by examiners who were unaware of the study protocol. Moreover, a set of in vitro experiments on isolated samples of canine lower oesophageal sphincter (LOS) was performed, and the effects of increasing concentrations of sildenafil on basal tone and electrically-stimulated motility were assessed. Sildenafil administration significantly reduced the number of regurgitation episodes (0.88±1.40 *vs* 2.65±1.56, P<0.0001) and increased weight gain in dogs significantly compared to controls (79.76±28.30% *vs* 53.40±19.30%, P=0.034). ROD values, at the end of the treatment period, were significantly decreased in dogs of sildenafil group, compared to pre-treatment values (0.97±0.19 *vs* 0.24±0.14, P<0.0001), contrary to control subjects (0.98±0.17 *vs* 1.10±0.25, P=0.480). In accordance with the in vivo findings, sildenafil dose-dependently reduced basal tone and increased electrically-induced relaxation of dog LOS samples. These results suggest that sildenafil citrate helps ameliorate clinical and radiographic signs in dogs with CIM by reducing LOS tone, and could represent a novel therapeutic tool for the treatment of this disease.

Introduction

The term megaoesophagus is used to describe a disease characterised by reduced or absent oesophageal motility which causes the accumulation of ingesta, dilatation of oesophageal lumen, food regurgitation (which is often mistaken for vomit by the dog owner), and weight loss as the main clinical signs. Megaoesophagus may be idiopathic, congenital or acquired, or secondary to different aetiologies, such as myasthenia gravis, hypothyroidism or Addison's disease. Congenital idiopathic megaoesophagus (CIM) is often observed at or before 10 weeks of life, and the condition frequently affects more than one animal in the same litter (Harvey and others 1974; Glidewell 1983).

CIM causes poor weight gain in puppies shortly after weaning, and, even though most animals tend to show spontaneous improvement over time, they require long-lasting physical and nutritional support, and the risk of fatal complications, like aspiration pneumonia, is high.

The pathogenesis of CIM is currently unclear. A predisposition for the disease has been reported in large and giant-breed dogs such as the German Shepherd, Great Dane, Irish Setter, Labrador Retriever, Irish Wolfhound and Newfoundland (Knowles and others 1990), and genetics might play a role in the aetiology of CIM, because autosomal dominant inheritance has been demonstrated in Miniature Schnauzers and Fox Terriers (Washabau 2003). A suspected hereditary form has also been reported in Bouvier des Flandres dogs (Peeters and others 1991).

It has been hypothesised that the congenital form of the disease is linked to a reduced or delayed development of the oesophageal neuromuscular system, in particular of the afferent vagal innervation, which fails to respond to the mechanical stimulus induced by food, thus resulting in ineffective peristalsis (Holland and others 1994, 1996, 2002). Manometric studies have found a normal tone and functioning of the lower oesophageal sphincter (LOS) in dogs with idiopathic megaoesophagus (Diamant and others 1973), unlike in other oesophageal motility disorders in humans, such as achalasia or diffuse oesophageal spasm, where a hypertonicity of sphincter muscle is present (Pohl and Tutuian 2007; Roman and Kahrilas 2012). However, a failure by LOS to relax

in response to intraluminal balloon distension was observed (Tan and Diamant 1987), thus further supporting the hypothesis of a functional defect of oesophageal sensory innervation.

CIM treatment is frustrating, resulting in high mortality from directly related causes like malnutrition and aspiration pneumonia or to euthanasia required because of the continuing clinical signs (Harvey and others 1974; McBrearty and others 2011). In the majority of cases, drugs are not adequately effective, and the treatment is based mostly on nutritional support and alterations in body position (Chandra and others 1989). Several pharmacological approaches, especially with prokinetic drugs such as metoclopramide, domperidone or cisapride, have been proposed, with modest or varying results (Washabau 2003). However, recent studies with high-resolution manometry showed that cisapride significantly increased LOS pressure in healthy dogs, and this could represent a serious concern in dogs with megaoesophagus (Kempf and others 2014; Ullal and others 2016).

Swallowing and oesophageal motility are complex processes involving a multifaceted interplay between excitatory innervation, mostly vagal cholinergic fibers, and inhibitory innervation, which releases nitric oxide (NO) as the main neurotransmitter. Endogenous NO induces smooth muscle relaxation through the synthesis of the second messenger cyclic guanosine monophosphate (cGMP). Sildenafil, a selective phosphodiesterase-type 5 (PDE-5) inhibitor, indirectly potentiates the action of endogenous NO by reducing cGMP degradation due to PDE-5 (Zhu and others 2007). Sildenafil is an effective vasodilator, widely prescribed for the treatment of erectile disorders in man, but it is also employed against pulmonary hypertension, and it relaxes smooth muscle of other organs, like the uterus (Méhats and others 2006), and the gallbladder (Bumin and others 2006). Vasorelaxant properties of sildenafil were also observed in dogs (Souza-Silva and others 2005; Bach and others 2006) and this drug represents a valid treatment option for pulmonary hypertension in this species.

Given that in humans and cats sildenafil has already been shown to induce the relaxation of LOS (Zhang and others 2001; Fox and others 2007), in the current study we evaluated the therapeutic efficacy of sildenafil in dogs affected by CIM, on the premise that a decreased LOS tone would

facilitate the entry of the ingesta into the stomach, thus reducing the pressure inside the oesophageal lumen. The effects of sildenafil were assessed by evaluating the clinical signs of the disease, and by means of oesophagrams. Moreover, in order to better understand the effects of sildenafil observed in vivo, a set of in vitro experiments on smooth muscle samples of canine LOS were also performed.

Materials and Methods

Animals

Twenty-one puppies of both sexes with clinical and radiographic signs of CIM from 6 breeding kennels were enroled in the study (Table 1). The sample size was determined on the basis of a previous study (Lee and others 2003).

CIM was initially suspected on the basis of patient age (≤ 50 days), history and clinical findings, such as post-prandial regurgitation of undigested food, palpable enlargement of the oesophagus, poor body condition, and the diagnosis was then confirmed by plain radiography and oesophagrams. Preadmission exclusion criteria included the presence of one or more of the following conditions: diarrhoea, cardiovascular abnormalities, distension of oesophagus limited to the cervical region, and clinical signs (fever, nasal discharge, cough) or radiographic evidence of aspiration pneumonia. None of the puppies had received any medication within 48 hours from the beginning of the study, and none were previously treated with prokinetic drugs.

The present study was conducted as a randomised controlled trial, therefore the dogs eligible for enrolment were randomly assigned to two parallel groups, treatment and control (placebo) group, with an allocation ratio of 1:1.

Informed consent about the nature of diagnostic and experimental procedures to be performed was obtained by dog owners (breeders), before enrolling their puppies. The trial was conducted in

compliance with institutional guidelines for reasearch on animals, and it was approved by the Ethics Committe of the University of Parma (O.P.B.A.), Prot. N. 136/OPBA/2016.

Each dog was given the daily amount of food, according to the caloric requirements of each breed, divided in 6 equal small meals. All dogs were fed with the same homogenised commercial canned puppy food from an elevated position, and none were managed with a percutaneous endoscopy gastrotomy tube. Moreover, all the dogs were kept in elevated position for 10 minutes after each meal, and carefully observed for the following 30 minutes, to detect possible regurgitation episodes. For each dog, the breeder was asked to randomly choose one of two identical bottles (labelled "A" and "B"), and therefore was masked to the nature of the content. Bottles labelled "A" contained sildenafil citrate (Revatio® 10 mg/ml oral suspension, Pfizer Italia), whereas the bottles labelled "B" contained placebo, i.e. a suspension prepared with only water and the excipients present in Revatio® commercial formulation, and with the same physical aspect. 0.1 ml/kg of suspension "A" or "B" was administered directly into the oral cavity, using a syringe, to the dogs, every 12 hours for two weeks, by a member of our research group, unaware of the treatment protocol. The dogs allocated in the treatment group received therefore 1 mg/kg sildenafil every 12 hours; this dose was chosen on the basis of previous studies in humans and cats (Bortolotti and others 2000; Zhang and others 2004), in which the same dose was effective in relaxing LOS. In order to obtain a prolonged effect on LOS tone, the drug was administered twice daily, since the half-life of sildenafil in dogs is about 5 hours (Walker and others 1999).

Dog owners were asked to record (on an appropriate data sheet) the frequency of regurgitation occurring within a 24 hours-period on different times: D0 (the day before the beginning of the treatment), D1 (1st day of treatment), D2 (2nd day of treatment), D3 (3rd day of treatment), D4 (4th day of treatment), D5 (5th day of treatment), D7 (7th day of treatment), D10 (10th day of treatment), D14 (14th day of treatment), D21 (7 days after the end of treatment), and D45 (30 days after the end of treatment) (Fig. 1). Dogs in both groups were weighed daily for the precise dose calculation, and the weight measured at D0, and the day after the last sildenafil or placebo administration (D15),

(named W1 and W2, respectively) were used to calculate the weight gain (WG) for each dog, expressed as percentage (Fig. 1). Dog owners were also asked to report immediately to the investigators any adverse event observed during or after the drug administration period. Apart from the regurgitation count, which took place in the breeding kennels, all the other evaluations were performed in the Veterinary Hospital facilities.

Radiographic evaluation

Lateral radiographs of each dog were taken, without any pharmacological restraint, before and immediately after the administration of 4 ml/kg of a barium suspension (Prontobario 60%, Bracco Imaging Italia, Milan), mixed with 3-4 boluses of canned food, without keeping the dog in lateral recumbency. Radiographic evaluation was performed at D0, and the day after the last sildenafil or placebo administration (D15). The oesophageal diameter (OD) was measured in each radiograph at its widest point, perpendicularly to the oesophageal longitudinal axis, at the level of its luminal surface. The thoracic inlet (TI) was also measured in the same radiograph, from the ventral aspect of the vertebral column at the mid-point of the first rib, to the inner aspect of the manubrium at the point of narrowest diameter of the TI. In order to minimise the differences in weight and size of the dogs in the two groups, the relative oesophageal diameter (ROD) was adopted instead of OD, using the function OD/TI, as proposed by Wray and Sparkes (2006) (Fig. 2). All measures were performed with an image analysis software (Image J, ver.1.49 NIH), by an examiner who was unaware of the study protocol.

In vitro experiments

Following laparatomy, the gastroesophageal junction was excised from six dogs of different breeds, euthanised at the Animal Hospital of the Department of Veterinary Science for reasons unrelated to pathologies of digestive system. Each segment of oesophagus was put in cooled (4 °C) modified Krebs-Henseleit Solution (KHS) of the following composition (mM): NaCl 113.0, KCl 4.7, $MgSO_4$

· $7H_2O$ 1.2, $CaCl_2$ · $2H_2O$ 1.8, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and dextrose 11.2, and immediately carried to the laboratory.

The tissue was cut longitudinally, pinned flat and the mucosa removed. Eight strips of circular smooth muscle (0.3-0.4 by 1.0-1.5 cm) were obtained from the LOS region. The strips were tied at each end with silk thread and set-up in organ baths (10 ml) filled with KHS, maintained at 37 °C and continuously bubbled with 95% O_2 and 5% CO_2. After a period of stabilization (45-60 min), the mechanical activity was measured by means of an isotonic transducer developing a passive load of 2–3 g to the preparation throughout the entire experiment. In a separate set of experiments, electrical field stimulation (EFS) was applied with a pair of coaxial platinum electrodes positioned 10 mm from the longitudinal axis of the preparation and used to deliver trains of square wave pulses (0.4 ms duration, 50 V amplitude) every 120 sec to the tissue at a frequency of 20 Hz. For each experiment, the intensity was adjusted to a level giving 70–80% of the maximum tissue response (usually 250–300 mA). Under these conditions, depolarisation of intrinsic nerve endings and neurotransmitter release were induced, as described previously (Poli and others 1994; Rakestraw and others 1996). All experiments were performed in presence of atropine (10^{-6} M), guanethidine (10^{-5} M), and indomethacin (10^{-5} M), to prevent the contractile effect evoked by endogenous acethylcholine, catecholamines, and prostaglandins, respectively. When concentration-response curves were needed, drugs were added cumulatively to the bath solution in 1 log unit increments of concentration. For in vitro experiments, sildenafil citrate 0.8 mg/ml (Revatio® i.v. solution, Pfizer Italia), was employed. The effect of drugs on basal tone was measured as the modification of the muscle length (Δ cm) with respect to the pre-drug level (baseline); the drug-induced variations of EFS-evoked responses of the preparation were expressed as a percentage of the pre-drug amplitude, assumed as 100%. All recordings were performed by means of a pen-writing polygraph (Basile, Milan, Italy).

Drugs

Sildenafil citrate pharmacological forms (Revatio® oral suspension, Revatio® i.v. solution) were purchased from Pfizer Italia; atropine, guanethidine, indomethacin, tetrodotoxin (TTX), L-NG-nitroarginine methyl ester (L-NAME), and 1H-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ) were purchased by Sigma (Sigma–Aldrich, St. Louis, MO, USA).

Statistical Analysis

Data are expressed as mean±sd. Unpaired t-tests were used for the comparison of data between the treatment and control groups, while paired t-tests were employed to compare pre- and post-treatment data in the same group. All analyses were performed using a commercial statistical software (GraphPad Prism for Mac ver.6.0f, GraphPad Software Inc., USA).

Results

The trial was conducted between November 2013 and January 2016. The treatment group (n=12) consisted of 7 Great Danes (four of which were littermates), 3 German Shepherds and 2 Labrador Retrievers. The control group (n=9) consisted of 5 Great Danes (two of which were littermates) and 4 German Shepherds. The mean ages of dogs were 28.17±6.07 days (range: 22-45) and 28.44±3.00 days (range: 25-35), in the treatment group and in the control group, respectively (P=0.389). Mean weight at the start of the study (W1) among the treatment group was 3.23±0.92 kg (range: 2–5.1 kg) whereas among the control group it was 3.63±0.92 kg (range: 2.8–5.2 kg), (P=0.453). The mean weight gain (WG) at D15 in the treatment group (79.76±28.30%) was significantly higher

(P=0.034) than the one measured in dogs of the control group (53.40±19.30%). The values of mean ages and weights in the two groups are shown in Table 1.

The owners did not observe regurgitation episodes within three hours after the administration of either "A" or "B" suspension. There was no significant difference in frequency of regurgitation between treatment and control group at D0 (P=0.540). The number of regurgitation episodes were markedly decreased in puppies of the treatment group after the first sildenafil dose (Table 1). Regurgitation episodes ceased almost completely after 10 days of sildenafil administration (D10), and no relapses were observed up to 1 month after the end of the treatment (D45). Conversely, regurgitation persisted in the control group, although a gradual reduction in frequency was noted with nutritional management alone (Table 1). Overall, puppies in the control group had more than two-fold total regurgitation episodes throughout the study period, compared to treatment group (262 vs 116). Mean regurgitation episode number in 24 hours was significantly lower in dog receiving sildenafil with respect to placebo-treated ones (0.88±1.40 *vs* 2.65±1.56, P<0.0001). No adverse effects were reported by the dog owners during the entire trial period.

Lateral thoracic radiograph measurements are shown in Figure 3. The mean ROD at D0 in the control group was 0.98±0.17 (range: 0.67–1.21), and in the treatment group it was 0.97±0.19 (range: 0.69-1.44), (P=0.663). The values of ROD at D15 in dogs of the treatment group were significantly lower (0.24±0.14) (range: 0.02-0.44), compared to the control group, in which the mean ROD value was 1.10±0.25 (range: 0.82-1.47) (P<0.0001). Sildenafil administration was also able to reduce mean oesophageal diameter in a significant fashion, as observed by comparing ROD values at D0 vs D15 (P<0.0001). By contrast, no significant difference was recorded in the control group between the ROD values at D0, with respect to D15 (P=0.480).

In the experiments performed in vitro, sildenafil (10^{-9}-10^{-5} M) induced a concentration-dependent decrease of basal tone of LOS preparations, as showed by the fall of the baseline with respect to pre-drug level (Fig. 4). In presence of atropine, guanethidine and indomethacin, EFS evoked non-

cholinergic non-adrenergic phasic relaxations of LOS muscle (Fig. 4), which were abolished by neuronal sodium channel blocker, TTX, by NO-synthase inhibitor, L-NAME, and by guanylyl cyclase inhibitor, ODQ. Sildenafil (10^{-10}-$3x10^{-6}$ M) enhanced the amplitude of these relaxations in a concentration-dependent fashion (Fig. 4).

Discussion

To date, there is no specific and effective pharmacological treatment for idiopathic megaoesophagus. In dogs, the oesophagus possesses a striated muscle layer in its entire length, excluding the LOS, therefore prokinetic agents which act on smooth muscle, such as metoclopramide and cisapride, are ineffective and could be contra-indicated (Washabau 2003). In accordance with this, 5-HT_4 serotonin receptors were not detected in oesophageal muscle of dogs (Cohen and others 1994). Moreover, metoclopramide and cisapride tend to increase LOS tone, further hindering the emptying of oesophageal content, and thus worsening the clinical signs (Washabau 1997). On the other hand, bethanechol, a muscarinic agonist, was instead shown to increase the amplitude of contractions in dogs with idiopathic megaoesophagus (Diamant and others 1974).

Due to the scarce results obtained with drugs aiming to enhance the contractions of the oesophageal body, a possible therapeutic strategy could be to relax LOS smooth muscle, in order to promote the emptying of the oesophagus. Indeed, calcium channel blockers were shown to be able to decrease LOS pressure in humans with oesophageal motor dysfunctions (Baunack and others 1991), and nifedipine administration resulted in a temporary clinical improvement in dogs with idiopathic megaoesophagus (Chandra and others 1989). A possible detrimental effect exerted by calcium antagonists on overall oesophagus peristalsis cannot be excluded, though, and it may represent a serious concern.

The importance of NO in basal and swallowing-induced LOS relaxation, as well as the ability of sildenafil to modify LOS tone, have been demonstrated several times in different species over the past two decades. For example, seminal work in the opossum demonstrated that the inhibition of NO synthesis antagonised swallowing-induced LOS relaxation, and caused an increase in basal LOS pressure (Tottrup and others 1991; Yamato and others 1992). Indeed, sildenafil was shown to decrease LOS tone in healthy humans or in patients with achalasia or other oesophageal motility disorders (Bortolotti and others 2000, 2002; Rhee and others 2001; Eherer and others 2002; Lee and others 2003; Fox and others 2007). An average basal LOS relaxation of 50% was also observed in sildenafil-treated cats (Zhang and others 2004).

The present study provides the first evidence documenting the benefits of sildenafil citrate in relieving the clinical signs associated to CIM in dogs. Although a decrease in the number of regurgitations from D0 to D14 was observed in both groups, it was markedly higher in the sildenafil group. Moreover, in the puppies treated with sildenafil, the mean frequency of regurgitation episodes in 24 h was significantly lower, compared to non-treated subjects, and the clinical improvement was supported by a significant increase of weight gain in the treatment group with respect to controls. The beneficial effects of the drug were also observed radiographically: in dogs of the treatment group, a marked reduction of the oesophageal diameter was measured at the end of the treatment period, as indicated by the significant lower mean ROD values, compared to control group. By contrast, in all the dogs enrolled in the control group the oesophageal diameter was wider at D15, with respect to the beginning of the study. In placebo-treated dogs a gradual decrease of regurgitation episodes was observed despite a worsening of oesophageal enlargement; although this discrepancy might seem surprising, there is usually poor correlation between the severity of clinical signs and the degree of oesophageal distension in dogs with megaoesophagus (Guilford 1990), and spontaneous improvement with time may be due to feeding from upright position (Sokolovsky 1972).

NO is the principal inhibitory neurotransmitter released from myenteric neurons which induces relaxation of the LOS, through activation of cGMP synthesis (Mittal and Bhalla 2004).

The importance of NO/cGMP pathway for the relaxation of LOS muscle in dogs was corroborated by the results of in vitro experiments, which evidenced that NO-synthase inhibitor L-NAME inhibited EFS-evoked relaxation spikes of LOS preparations, in accordance with what was observed previously (Yamato and others 1992). Moreover, ODQ, a guanylyl cyclase inhibitor, abolished such relaxations, confirming that they were mediated by cGMP, and thus could be susceptible to sildenafil action. Actually, sildenafil, enhanced EFS-evoked relaxation spikes and reduced basal tone in a concentration-dependent manner, showing that this PDE-5 inhibitor is able to induce the relaxation of isolated LOS in the dog. These results strongly support the hypothesis that the clinical and radiographic improvement observed in dogs treated with sildenafil are indeed due to a reduced LOS tone, with subsequent easier transit of food from the oesophagus into the stomach.

The ability of sildenafil to relax smooth muscle could also represent a concern, though, as it might furtherly hinder oesophageal peristalsis. As a matter of fact, in previous studies in humans and in cats, sildenafil significantly reduced oesophageal contractile pressures (Bortolotti and others 2000, 2002; Eherer and others 2002; Zhang and others 2004). Unlike in human and cats, however, oesophageal muscle in dogs is almost entirely of the striated type, and thus not affected by sildenafil effects. Indeed, the work by Zhang and others showed that the contractile amplitude in oesophageal portions with striated muscle was unaffected. Another concern of the reduced tone of LOS induced by sildenafil could be represented by a potential increased risk of gastro-oesophageal reflux (GOR); a previous study, though, found that sildenafil is altering LOS function without causing GOR in human patients (Kim and others 2006).

Since peristalsis of the oesophagus is unchanged, though, the dogs affected by CIM treated with sildenafil would still require to be fed from an elevated position, but could take great advantage from the easier oesophagus emptying and the decrease of oesophagus dilatation, which result in an improvement of clinical signs and general health status. Moreover, serious complications like

aspiration pneumonia are less likely to occur. Interestingly, sildenafil seemed to achieve results that go beyond a mere symptomatic treatment, since puppies in the sildenafil group had only occasional regurgitation episodes up to 30 days after the drug administration was discontinued, whereas the clinical signs, though improved, were considerably worse in the control subjects. CIM is a chronic disease, so it would be very important in future studies to expand the knowledge about sildenafil effects over time. Further experiments with different doses of sildenafil and with similar drugs, like tadalafil, will be necessary for a better understanding of PDE-5 inhibitors efficacy against idiopathic megaoesophagus in dogs. Moreover, gastro-oesophageal manometric studies should be performed to enlighten sildenafil activity on oesophageal and LOS tone and contractility.

The current dosage was well tolerated in all treated puppies. Aside from the possible decrease of blood pressure, several adverse reactions have been reported in literature after sildenafil administration. Abbott and others (2004), for example, described species-specific effects in dogs (Beagle pain syndrome), mice and rats. For this reason, additional clinical studies in dogs would benefit from arterial pressure measurement, urinalysis, haematological and serum biochemical analyses in sildenafil-treated patients.

In conclusion, this preliminary study suggests, for the first time, that sildenafil citrate, by reducing LOS tone and facilitating the emptying of the oesophagus, could represent a useful drug for the clinical management of CIM in dogs.

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	weight assessment			number of regurgitation episodes in 24 hours									
	W1 (kg)	**W2 (kg)**	**WG (%)**	**D0**	**D1**	**D2**	**D3**	**D4**	**D5**	**D7**	**D10**	**D14**	**D21**	**D45**
Control group	3.63 ± 0.92	5.45 ± 0.86	53.40 ± 19.30	5.00 ± 0.86	4.56 ± 1.33	3.44 ± 0.88	3.33 ± 0.87	3.00 ± 1.00	2.11 ± 0.93	2.00 ± 1.00	2.33 ± 0.71	1.44 ± 0.73	1.11 ± 0.78	0.78 ± 0.67
Treatment group	3.23 ± 0.92	5.60 ± 0.84	79.76 ± 28.30 *	4.58 ± 1.24	1.58 ± 0.90	0.83 ± 0.72	0.33 ± 0.49	0.67 ± 0.49	0.42 ± 0.90	0.50 ± 0.52	0.25 ± 0.45	0.17 ± 0.39	0.17 ± 0.39	0.17 ± 0.39

Table 1. Weight values of dogs in control and treatment group recorded at day 0 (W1) and at day 15 (W2), and weight gain (WG). Regurgitation frequency recorded in a 24 hours-period at different times. D=day number. All values are expressed as mean±sd. *P=0.034 treatment *vs* control group.

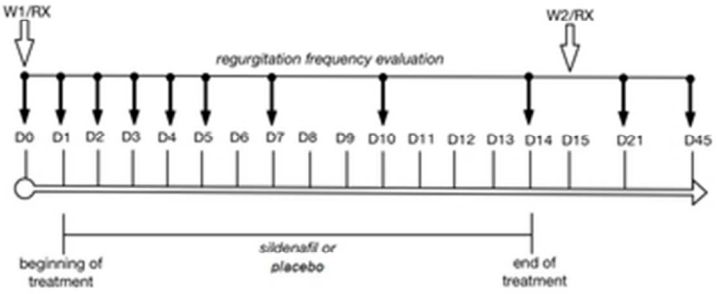
Figure legends

Figure 1. Study design scheme indicating: the duration of the study (from day 0 to day 45, D0-D45); sildenafil (1 mg/kg bid) or placebo administration protocol; times of regurgitation frequency evaluation; weight recordings (W1 and W2), and radiographic evaluation times (RX).

Figure 2. Radiographic measurement technique as proposed by Wray and Sparkes (2007). The oesophageal diameter (OD) was measured in each radiograph at its widest point, perpendicularly to the oesophageal longitudinal axis, at the level of its luminal surface. The thoracic inlet (TI) was also measured in the same radiograph, from the ventral aspect of the vertebral column at the mid-point of the first rib, to the inner aspect of the manubrium at the point of narrowest diameter of the TI. The relative oesophageal diameter (ROD) was calculated, using the OD/TI ratio.

Figure 3. Mean±sd of ROD (OD/TI) values measured at day 0 (D0) and day 15 (D15) for control group (Co) and treatment group (Sil). **P<0.0001 Sil D15 *vs* Co D15, and $^{\#\#}$P<0.0001 Sil D15 *vs* Sil D0.

Figure 4. Effect of sildenafil (10^{-9}-10^{-5} M) on basal tone (baseline) of dog LOS (panel A). Effects of L-NAME (10^{-6}-10^{-3} M), TTX (10^{-6} M), and ODQ (10^{-6}-10^{-4} M) on the amplitude of EFS-evoked relaxation spikes of dog LOS (panel B). Effect of sildenafil (10^{-10}-10^{-5} M) on the amplitude of EFS-evoked relaxation spikes of dog LOS (panel C). All the experiments were performed in presence of atropine (10^{-6} M), guanethidine (10^{-5} M), and indomethacin (10^{-5} M) (not shown). Data represent mean±sd of 8 experiments.



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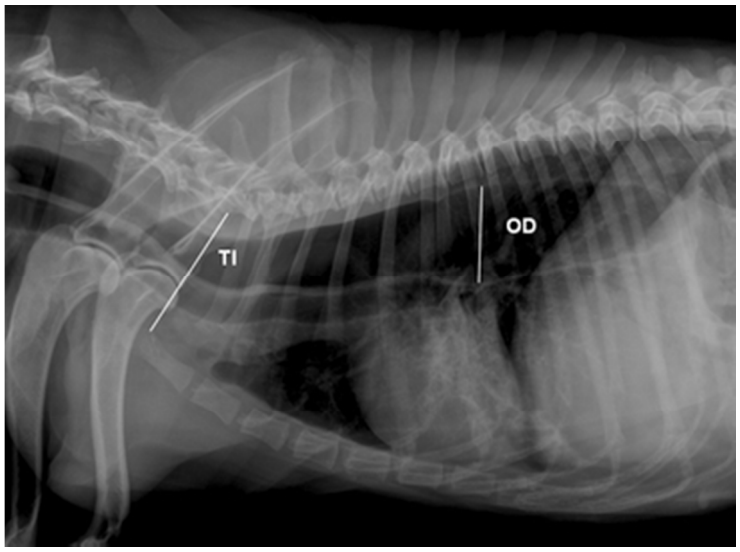

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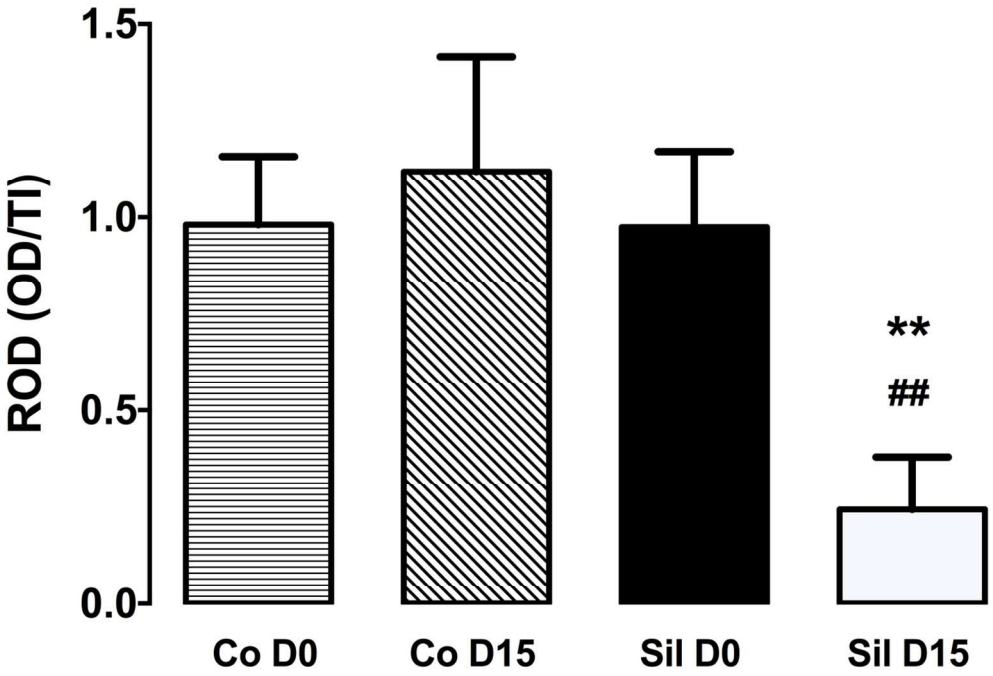

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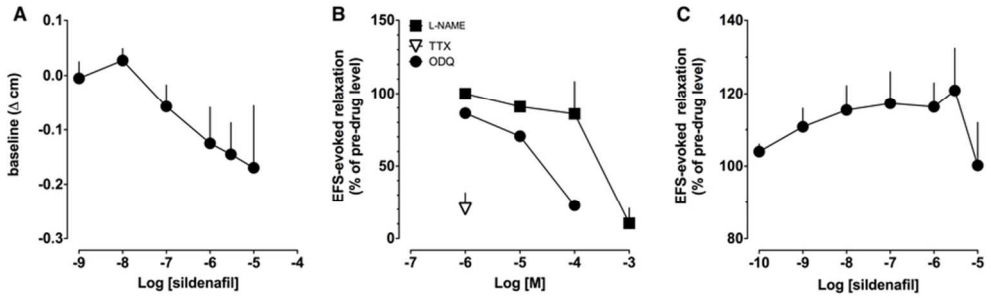

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78x24mm (300 x 300 DPI)