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

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

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Abstract:	<p>We evaluated the efficacy of oral sildenafil citrate in dogs with congenital idiopathic megaesophagus (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 (0.881.40 vs 2.651.56, $P < 0.0001$) and increased weight gain in dogs significantly compared to controls (79.7628.30% vs 53.4019.30%, $P = 0.034$). ROD values, at the end of the treatment period, were significantly decreased in dogs of sildenafil group, compared to pre-treatment values (0.970.19 vs 0.240.14, $P < 0.0001$), contrary to control subjects (0.980.17 vs 1.100.25, $P = 0.480$). 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.</p>

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3 1 **SILDENAFIL IMPROVES CLINICAL SIGNS AND RADIOGRAPHIC**
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5 2 **FEATURES IN DOGS WITH CONGENITAL IDIOPATHIC**
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7 3 **MEGAESOPHAGUS: A RANDOMISED CONTROLLED TRIAL**
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11 4 **F. Quintavalla, A. Menozzi*, C. Pozzoli, E. Poli, P. Donati, D.K. Wyler, P. Serventi, S. Bertini**
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28 **Abstract**

29 We evaluated the efficacy of oral sildenafil citrate in dogs with congenital idiopathic
30 megaesophagus (CIM). Twenty-one puppies were randomly assigned to two groups (treatment and
31 control). The dogs were given sildenafil oral suspension 1 mg/kg every 12 hours for 14 days or
32 placebo in a masked fashion. Clinical signs (frequency of regurgitation and weight gain) and
33 oesophagrams (relative oesophageal diameter, ROD) were evaluated in order to assess the efficacy
34 of drug treatment, by examiners who were unaware of the study protocol. Moreover, a set of in vitro
35 experiments on isolated samples of canine lower oesophageal sphincter (LOS) was performed, and
36 the effects of increasing concentrations of sildenafil on basal tone and electrically-stimulated
37 motility were assessed. Sildenafil administration significantly reduced the number of regurgitation
38 episodes (0.88 ± 1.40 vs 2.65 ± 1.56 , $P < 0.0001$) and increased weight gain in dogs significantly
39 compared to controls ($79.76 \pm 28.30\%$ vs $53.40 \pm 19.30\%$, $P = 0.034$). ROD values, at the end of the
40 treatment period, were significantly decreased in dogs of sildenafil group, compared to pre-
41 treatment values (0.97 ± 0.19 vs 0.24 ± 0.14 , $P < 0.0001$), contrary to control subjects (0.98 ± 0.17 vs
42 1.10 ± 0.25 , $P = 0.480$). In accordance with the in vivo findings, sildenafil dose-dependently reduced
43 basal tone and increased electrically-induced relaxation of dog LOS samples. These results suggest
44 that sildenafil citrate helps ameliorate clinical and radiographic signs in dogs with CIM by reducing
45 LOS tone, and could represent a novel therapeutic tool for the treatment of this disease.

48 Introduction

49 The term megaesophagus is used to describe a disease characterised by reduced or absent
50 oesophageal motility which causes the accumulation of ingesta, dilatation of oesophageal lumen,
51 food regurgitation (which is often mistaken for vomit by the dog owner), and weight loss as the
52 main clinical signs. Megaesophagus may be idiopathic, congenital or acquired, or secondary to
53 different aetiologies, such as myasthenia gravis, hypothyroidism or Addison's disease. Congenital
54 idiopathic megaesophagus (CIM) is often observed at or before 10 weeks of life, and the condition
55 frequently affects more than one animal in the same litter (Harvey and others 1974; Glidewell
56 1983).

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

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

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

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3 74 in response to intraluminal balloon distension was observed (Tan and Diamant 1987), thus further
4
5 75 supporting the hypothesis of a functional defect of oesophageal sensory innervation.
6
7 76 CIM treatment is frustrating, resulting in high mortality from directly related causes like
8
9 77 malnutrition and aspiration pneumonia or to euthanasia required because of the continuing clinical
10
11 78 signs (Harvey and others 1974; McBrearty and others 2011). In the majority of cases, drugs are not
12
13 79 adequately effective, and the treatment is based mostly on nutritional support and alterations in
14
15 80 body position (Chandra and others 1989). Several pharmacological approaches, especially with
16
17 81 prokinetic drugs such as metoclopramide, domperidone or cisapride, have been proposed, with
18
19 82 modest or varying results (Washabau 2003). However, recent studies with high-resolution
20
21 83 manometry showed that cisapride significantly increased LOS pressure in healthy dogs, and this
22
23 84 could represent a serious concern in dogs with megaesophagus (Kempf and others 2014; Ullal and
24
25 85 others 2016).
26
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28
29 86 Swallowing and oesophageal motility are complex processes involving a multifaceted interplay
30
31 87 between excitatory innervation, mostly vagal cholinergic fibers, and inhibitory innervation, which
32
33 88 releases nitric oxide (NO) as the main neurotransmitter. Endogenous NO induces smooth muscle
34
35 89 relaxation through the synthesis of the second messenger cyclic guanosine monophosphate (cGMP).
36
37 90 Sildenafil, a selective phosphodiesterase-type 5 (PDE-5) inhibitor, indirectly potentiates the action
38
39 91 of endogenous NO by reducing cGMP degradation due to PDE-5 (Zhu and others 2007). Sildenafil
40
41 92 is an effective vasodilator, widely prescribed for the treatment of erectile disorders in man, but it is
42
43 93 also employed against pulmonary hypertension, and it relaxes smooth muscle of other organs, like
44
45 94 the uterus (Méhats and others 2006), and the gallbladder (Bumin and others 2006). Vasorelaxant
46
47 95 properties of sildenafil were also observed in dogs (Souza-Silva and others 2005; Bach and others
48
49 96 2006) and this drug represents a valid treatment option for pulmonary hypertension in this species.
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52 97 Given that in humans and cats sildenafil has already been shown to induce the relaxation of LOS
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54 98 (Zhang and others 2001; Fox and others 2007), in the current study we evaluated the therapeutic
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56 99 efficacy of sildenafil in dogs affected by CIM, on the premise that a decreased LOS tone would
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3 100 facilitate the entry of the ingesta into the stomach, thus reducing the pressure inside the oesophageal
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5 101 lumen. The effects of sildenafil were assessed by evaluating the clinical signs of the disease, and by
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7 102 means of oesophagrams. Moreover, in order to better understand the effects of sildenafil observed
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9 103 in vivo, a set of in vitro experiments on smooth muscle samples of canine LOS were also
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11 104 performed.
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17 18 107 **Materials and Methods**

19 20 21 108 **Animals**

22
23 109 Twenty-one puppies of both sexes with clinical and radiographic signs of CIM from 6 breeding
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25 110 kennels were enrolled in the study (Table 1). The sample size was determined on the basis of a
26
27 111 previous study (Lee and others 2003).
28

29
30 112 CIM was initially suspected on the basis of patient age (≤ 50 days), history and clinical findings,
31
32 113 such as post-prandial regurgitation of undigested food, palpable enlargement of the oesophagus,
33
34 114 poor body condition, and the diagnosis was then confirmed by plain radiography and
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36 115 oesophagrams. Preadmission exclusion criteria included the presence of one or more of the
37
38 116 following conditions: diarrhoea, cardiovascular abnormalities, distension of oesophagus limited to
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40 117 the cervical region, and clinical signs (fever, nasal discharge, cough) or radiographic evidence of
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42 118 aspiration pneumonia. None of the puppies had received any medication within 48 hours from the
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44 119 beginning of the study, and none were previously treated with prokinetic drugs.
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46

47 120 The present study was conducted as a randomised controlled trial, therefore the dogs eligible for
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49 121 enrolment were randomly assigned to two parallel groups, treatment and control (placebo) group,
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51 122 with an allocation ratio of 1:1.
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54 123 Informed consent about the nature of diagnostic and experimental procedures to be performed was
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56 124 obtained by dog owners (breeders), before enrolling their puppies. The trial was conducted in
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3 125 compliance with institutional guidelines for reasearch on animals, and it was approved by the Ethics
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5 126 Committe of the University of Parma (O.P.B.A.), Prot. N. 136/OPBA/2016.
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7 127 Each dog was given the daily amount of food, according to the caloric requirements of each breed,
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9 128 divided in 6 equal small meals. All dogs were fed with the same homogenised commercial canned
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11 129 puppy food from an elevated position, and none were managed with a percutaneous endoscopy
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13 130 gastrotomy tube. Moreover, all the dogs were kept in elevated position for 10 minutes after each
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15 131 meal, and carefully observed for the following 30 minutes, to detect possible regurgitation episodes.
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17 132 For each dog, the breeder was asked to randomly choose one of two identical bottles (labelled "A"
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19 133 and "B"), and therefore was masked to the nature of the content. Bottles labelled "A" contained
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21 134 sildenafil citrate (Revatio® 10 mg/ml oral suspension, Pfizer Italia), whereas the bottles labelled "B"
22
23 135 contained placebo, i.e. a suspension prepared with only water and the excipients present in Revatio®
24
25 136 commercial formulation, and with the same physical aspect. 0.1 ml/kg of suspension "A" or "B"
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27 137 was administered directly into the oral cavity, using a syringe, to the dogs, every 12 hours for two
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29 138 weeks, by a member of our research group, unaware of the treatment protocol. The dogs allocated
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31 139 in the treatment group received therefore 1 mg/kg sildenafil every 12 hours; this dose was chosen
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33 140 on the basis of previous studies in humans and cats (Bortolotti and others 2000; Zhang and others
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35 141 2004), in which the same dose was effective in relaxing LOS. In order to obtain a prolonged effect
36
37 142 on LOS tone, the drug was administered twice daily, since the half-life of sildenafil in dogs is about
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39 143 5 hours (Walker and others 1999).
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41 144 Dog owners were asked to record (on an appropriate data sheet) the frequency of regurgitation
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43 145 occurring within a 24 hours-period on different times: D0 (the day before the beginning of the
44
45 146 treatment), D1 (1st day of treatment), D2 (2nd day of treatment), D3 (3rd day of treatment), D4 (4th
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47 147 day of treatment), D5 (5th day of treatment), D7 (7th day of treatment), D10 (10th day of treatment),
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49 148 D14 (14th day of treatment), D21 (7 days after the end of treatment), and D45 (30 days after the end
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51 149 of treatment) (Fig. 1). Dogs in both groups were weighed daily for the precise dose calculation, and
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53 150 the weight measured at D0, and the day after the last sildenafil or placebo administration (D15),
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3 151 (named W1 and W2, respectively) were used to calculate the weight gain (WG) for each dog,
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5 152 expressed as percentage (Fig. 1). Dog owners were also asked to report immediately to the
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7 153 investigators any adverse event observed during or after the drug administration period. Apart from
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9 154 the regurgitation count, which took place in the breeding kennels, all the other evaluations were
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11 155 performed in the Veterinary Hospital facilities.
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157 **Radiographic evaluation**

158 Lateral radiographs of each dog were taken, without any pharmacological restraint, before and
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20 159 immediately after the administration of 4 ml/kg of a barium suspension (Prontobario 60%, Bracco
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22 160 Imaging Italia, Milan), mixed with 3-4 boluses of canned food, without keeping the dog in lateral
23
24 161 recumbency. Radiographic evaluation was performed at D0, and the day after the last sildenafil or
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26 162 placebo administration (D15). The oesophageal diameter (OD) was measured in each radiograph at
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28 163 its widest point, perpendicularly to the oesophageal longitudinal axis, at the level of its luminal
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30 164 surface. The thoracic inlet (TI) was also measured in the same radiograph, from the ventral aspect
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32 165 of the vertebral column at the mid-point of the first rib, to the inner aspect of the manubrium at the
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34 166 point of narrowest diameter of the TI. In order to minimise the differences in weight and size of the
35
36 167 dogs in the two groups, the relative oesophageal diameter (ROD) was adopted instead of OD, using
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38 168 the function OD/TI , as proposed by Wray and Sparkes (2006) (Fig. 2). All measures were
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40 169 performed with an image analysis software (Image J, ver.1.49 NIH), by an examiner who was
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42 170 unaware of the study protocol.
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172 **In vitro experiments**

173 Following laparotomy, the gastroesophageal junction was excised from six dogs of different breeds,
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54 174 euthanised at the Animal Hospital of the Department of Veterinary Science for reasons unrelated to
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56 175 pathologies of digestive system. Each segment of oesophagus was put in cooled (4 °C) modified
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58 176 Krebs-Henseleit Solution (KHS) of the following composition (mM): NaCl 113.0, KCl 4.7, MgSO₄
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3 177 · 7H₂O 1.2, CaCl₂ · 2H₂O 1.8, KH₂PO₄ 1.2, NaHCO₃ 25.0 and dextrose 11.2, and immediately
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5 178 carried to the laboratory.

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7 179 The tissue was cut longitudinally, pinned flat and the mucosa removed. Eight strips of circular
8
9 180 smooth muscle (0.3-0.4 by 1.0-1.5 cm) were obtained from the LOS region. The strips were tied at
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11 181 each end with silk thread and set-up in organ baths (10 ml) filled with KHS, maintained at 37 °C
12
13 182 and continuously bubbled with 95% O₂ and 5% CO₂. After a period of stabilization (45-60 min), the
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15 183 mechanical activity was measured by means of an isotonic transducer developing a passive load of
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17 184 2–3 g to the preparation throughout the entire experiment. In a separate set of experiments,
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19 185 electrical field stimulation (EFS) was applied with a pair of coaxial platinum electrodes positioned
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21 186 10 mm from the longitudinal axis of the preparation and used to deliver trains of square wave pulses
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23 187 (0.4 ms duration, 50 V amplitude) every 120 sec to the tissue at a frequency of 20 Hz. For each
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25 188 experiment, the intensity was adjusted to a level giving 70–80% of the maximum tissue response
26
27 189 (usually 250–300 mA). Under these conditions, depolarisation of intrinsic nerve endings and
28
29 190 neurotransmitter release were induced, as described previously (Poli and others 1994; Rakestraw
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31 191 and others 1996). All experiments were performed in presence of atropine (10⁻⁶ M), guanethidine
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33 192 (10⁻⁵ M), and indomethacin (10⁻⁵ M), to prevent the contractile effect evoked by endogenous
34
35 193 acetylcholine, catecholamines, and prostaglandins, respectively. When concentration-response
36
37 194 curves were needed, drugs were added cumulatively to the bath solution in 1 log unit increments of
38
39 195 concentration. For in vitro experiments, sildenafil citrate 0.8 mg/ml (Revatio[®] i.v. solution, Pfizer
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41 196 Italia), was employed. The effect of drugs on basal tone was measured as the modification of the
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43 197 muscle length (Δ cm) with respect to the pre-drug level (baseline); the drug-induced variations of
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45 198 EFS-evoked responses of the preparation were expressed as a percentage of the pre-drug amplitude,
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47 199 assumed as 100%. All recordings were performed by means of a pen-writing polygraph (Basile,
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49 200 Milan, Italy).

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203 Drugs

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

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210 Statistical Analysis

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

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217 Results

218

219 The trial was conducted between November 2013 and January 2016. The treatment group (n=12)
220 consisted of 7 Great Danes (four of which were littermates), 3 German Shepherds and 2 Labrador
221 Retrievers. The control group (n=9) consisted of 5 Great Danes (two of which were littermates) and
222 4 German Shepherds. The mean ages of dogs were 28.17±6.07 days (range: 22-45) and 28.44±3.00
223 days (range: 25-35), in the treatment group and in the control group, respectively (P=0.389). Mean
224 weight at the start of the study (W1) among the treatment group was 3.23±0.92 kg (range: 2–5.1 kg)
225 whereas among the control group it was 3.63±0.92 kg (range: 2.8–5.2 kg), (P=0.453). The mean
226 weight gain (WG) at D15 in the treatment group (79.76±28.30%) was significantly higher

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

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

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

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

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3 252 cholinergic non-adrenergic phasic relaxations of LOS muscle (Fig. 4), which were abolished by
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5 253 neuronal sodium channel blocker, TTX, by NO-synthase inhibitor, L-NAME, and by guanylyl
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7 254 cyclase inhibitor, ODQ. Sildenafil (10^{-10} - 3×10^{-6} M) enhanced the amplitude of these relaxations in a
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9 255 concentration-dependent fashion (Fig. 4).
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12 257

13 258 **Discussion**

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16 260 To date, there is no specific and effective pharmacological treatment for idiopathic
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18 261 megaesophagus. In dogs, the oesophagus possesses a striated muscle layer in its entire length,
19
20 262 excluding the LOS, therefore prokinetic agents which act on smooth muscle, such as
21
22 263 metoclopramide and cisapride, are ineffective and could be contra-indicated (Washabau 2003). In
23
24 264 accordance with this, 5-HT₄ serotonin receptors were not detected in oesophageal muscle of dogs
25
26 265 (Cohen and others 1994). Moreover, metoclopramide and cisapride tend to increase LOS tone,
27
28 266 further hindering the emptying of oesophageal content, and thus worsening the clinical signs
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30 267 (Washabau 1997). On the other hand, bethanechol, a muscarinic agonist, was instead shown to
31
32 268 increase the amplitude of contractions in dogs with idiopathic megaesophagus (Diamant and others
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34 269 1974).

35
36 270 Due to the scarce results obtained with drugs aiming to enhance the contractions of the oesophageal
37
38 271 body, a possible therapeutic strategy could be to relax LOS smooth muscle, in order to promote the
39
40 272 emptying of the oesophagus. Indeed, calcium channel blockers were shown to be able to decrease
41
42 273 LOS pressure in humans with oesophageal motor dysfunctions (Baunack and others 1991), and
43
44 274 nifedipine administration resulted in a temporary clinical improvement in dogs with idiopathic
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46 275 megaesophagus (Chandra and others 1989). A possible detrimental effect exerted by calcium
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48 276 antagonists on overall oesophagus peristalsis cannot be excluded, though, and it may represent a
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50 277 serious concern.
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3 278 The importance of NO in basal and swallowing-induced LOS relaxation, as well as the ability of
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5 279 sildenafil to modify LOS tone, have been demonstrated several times in different species over the
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7 280 past two decades. For example, seminal work in the opossum demonstrated that the inhibition of
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9 281 NO synthesis antagonised swallowing-induced LOS relaxation, and caused an increase in basal
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11 282 LOS pressure (Tottrup and others 1991; Yamato and others 1992). Indeed, sildenafil was shown to
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13 283 decrease LOS tone in healthy humans or in patients with achalasia or other oesophageal motility
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15 284 disorders (Bortolotti and others 2000, 2002; Rhee and others 2001; Eherer and others 2002; Lee and
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17 285 others 2003; Fox and others 2007). An average basal LOS relaxation of 50% was also observed in
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19 286 sildenafil-treated cats (Zhang and others 2004).

22
23 287 The present study provides the first evidence documenting the benefits of sildenafil citrate in
24
25 288 relieving the clinical signs associated to CIM in dogs. Although a decrease in the number of
26
27 289 regurgitations from D0 to D14 was observed in both groups, it was markedly higher in the sildenafil
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29 290 group. Moreover, in the puppies treated with sildenafil, the mean frequency of regurgitation
30
31 291 episodes in 24 h was significantly lower, compared to non-treated subjects, and the clinical
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33 292 improvement was supported by a significant increase of weight gain in the treatment group with
34
35 293 respect to controls. The beneficial effects of the drug were also observed radiographically: in dogs
36
37 294 of the treatment group, a marked reduction of the oesophageal diameter was measured at the end of
38
39 295 the treatment period, as indicated by the significant lower mean ROD values, compared to control
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41 296 group. By contrast, in all the dogs enrolled in the control group the oesophageal diameter was wider
42
43 297 at D15, with respect to the beginning of the study. In placebo-treated dogs a gradual decrease of
44
45 298 regurgitation episodes was observed despite a worsening of oesophageal enlargement; although this
46
47 299 discrepancy might seem surprising, there is usually poor correlation between the severity of clinical
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49 300 signs and the degree of oesophageal distension in dogs with megaesophagus (Guilford 1990), and
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51 301 spontaneous improvement with time may be due to feeding from upright position (Sokolovsky
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53 302 1972).

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3 303 NO is the principal inhibitory neurotransmitter released from myenteric neurons which induces
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5 304 relaxation of the LOS, through activation of cGMP synthesis (Mittal and Bhalla 2004).

6
7 305 The importance of NO/cGMP pathway for the relaxation of LOS muscle in dogs was corroborated
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9 306 by the results of in vitro experiments, which evidenced that NO-synthase inhibitor L-NAME
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11 307 inhibited EFS-evoked relaxation spikes of LOS preparations, in accordance with what was observed
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13 308 previously (Yamato and others 1992). Moreover, ODQ, a guanylyl cyclase inhibitor, abolished such
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15 309 relaxations, confirming that they were mediated by cGMP, and thus could be susceptible to
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17 310 sildenafil action. Actually, sildenafil, enhanced EFS-evoked relaxation spikes and reduced basal
18
19 311 tone in a concentration-dependent manner, showing that this PDE-5 inhibitor is able to induce the
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21 312 relaxation of isolated LOS in the dog. These results strongly support the hypothesis that the clinical
22
23 313 and radiographic improvement observed in dogs treated with sildenafil are indeed due to a reduced
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25 314 LOS tone, with subsequent easier transit of food from the oesophagus into the stomach.

26
27 315 The ability of sildenafil to relax smooth muscle could also represent a concern, though, as it might
28
29 316 furtherly hinder oesophageal peristalsis. As a matter of fact, in previous studies in humans and in
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31 317 cats, sildenafil significantly reduced oesophageal contractile pressures (Bortolotti and others 2000,
32
33 318 2002; Eherer and others 2002; Zhang and others 2004). Unlike in human and cats, however,
34
35 319 oesophageal muscle in dogs is almost entirely of the striated type, and thus not affected by sildenafil
36
37 320 effects. Indeed, the work by Zhang and others showed that the contractile amplitude in oesophageal
38
39 321 portions with striated muscle was unaffected. Another concern of the reduced tone of LOS induced
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41 322 by sildenafil could be represented by a potential increased risk of gastro-oesophageal reflux (GOR);
42
43 323 a previous study, though, found that sildenafil is altering LOS function without causing GOR in
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45 324 human patients (Kim and others 2006).

46
47 325 Since peristalsis of the oesophagus is unchanged, though, the dogs affected by CIM treated with
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49 326 sildenafil would still require to be fed from an elevated position, but could take great advantage
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51 327 from the easier oesophagus emptying and the decrease of oesophagus dilatation, which result in an
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53 328 improvement of clinical signs and general health status. Moreover, serious complications like
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3 329 aspiration pneumonia are less likely to occur. Interestingly, sildenafil seemed to achieve results that
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5 330 go beyond a mere symptomatic treatment, since puppies in the sildenafil group had only occasional
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7 331 regurgitation episodes up to 30 days after the drug administration was discontinued, whereas the
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9 332 clinical signs, though improved, were considerably worse in the control subjects. CIM is a chronic
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11 333 disease, so it would be very important in future studies to expand the knowledge about sildenafil
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13 334 effects over time. Further experiments with different doses of sildenafil and with similar drugs, like
14
15 335 tadalafil, will be necessary for a better understanding of PDE-5 inhibitors efficacy against idiopathic
16
17 336 megaesophagus in dogs. Moreover, gastro-oesophageal manometric studies should be performed
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19 337 to enlighten sildenafil activity on oesophageal and LOS tone and contractility.

20
21 338 The current dosage was well tolerated in all treated puppies. Aside from the possible decrease of
22
23 339 blood pressure, several adverse reactions have been reported in literature after sildenafil
24
25 340 administration. Abbott and others (2004), for example, described species-specific effects in dogs
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27 341 (Beagle pain syndrome), mice and rats. For this reason, additional clinical studies in dogs would
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29 342 benefit from arterial pressure measurement, urinalysis, haematological and serum biochemical
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31 343 analyses in sildenafil-treated patients.

32
33 344 In conclusion, this preliminary study suggests, for the first time, that sildenafil citrate, by reducing
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35 345 LOS tone and facilitating the emptying of the oesophagus, could represent a useful drug for the
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37 346 clinical management of CIM in dogs.

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43 44 45 348 **Acknowledgements**

46
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51 351 2013).

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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
<i>Control group</i>	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
<i>Treatment group</i>	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.

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3 528 **Figure legends**

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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.

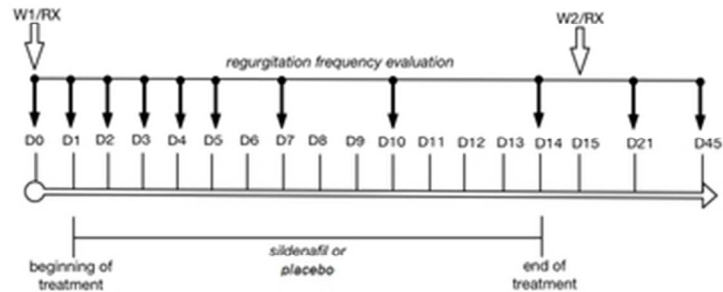


Figure 1. Study design scheme indicating: the duration of the study (from day 0 to day 45, D0-D45); sildenafil (1 mg/kg bid) or placebo administration protocol; times of regurgitation frequency evaluation; weight recordings (W1 and W2), and radiographic evaluation times (RX).

32x24mm (300 x 300 DPI)

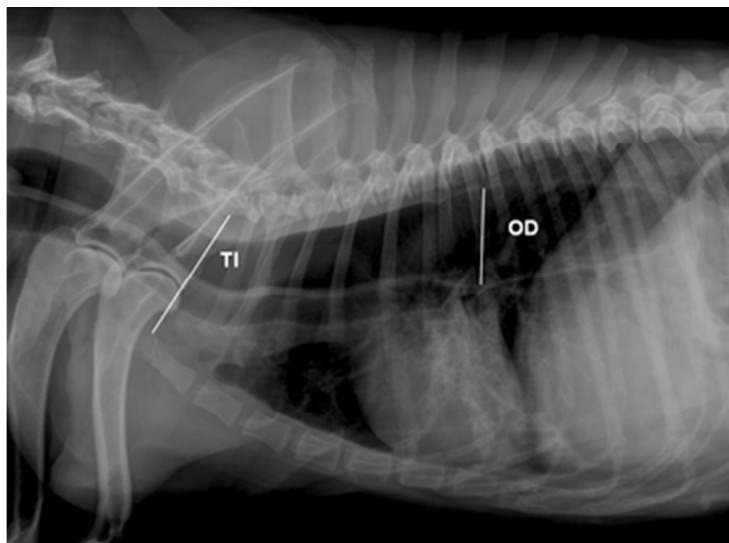


Figure 2. Radiographic measurement technique as proposed by Wray and Sparkes (2007). The oesophageal diameter (OD) was measured in each radiograph at its widest point, perpendicularly to the oesophageal longitudinal axis, at the level of its luminal surface. The thoracic inlet (TI) was also measured 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.

30x22mm (300 x 300 DPI)

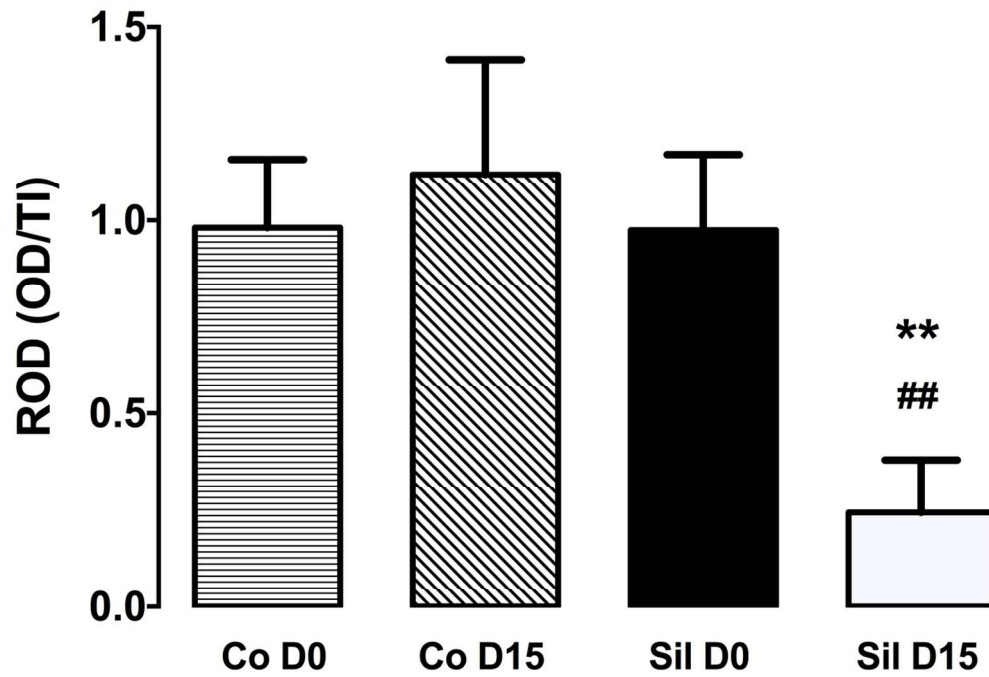


Figure 3. Mean±sd of ROD (OD/TI) values measured at day 0 (D0) and day 15 (D15) for control group (Co) and treatment group (Sil). ** $P < 0.0001$ Sil D15 vs Co D15, and ## $P < 0.0001$ Sil D15 vs Sil D0.

120x85mm (300 x 300 DPI)

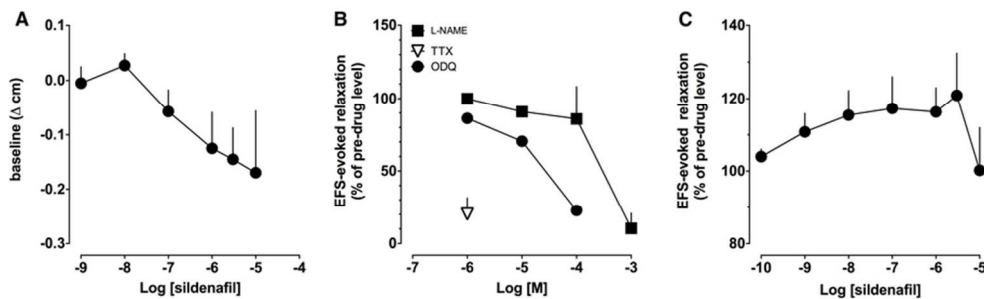


Figure 4. Effect of sildenafil (10⁻⁹-10⁻⁵ M) on basal tone (baseline) of dog LOS (panel A). Effects of L-NAME (10⁻⁶-10⁻³ M), TTX (10⁻⁶ M), and ODQ (10⁻⁶-10⁻⁴ M) on the amplitude of EFS-evoked relaxation spikes of dog LOS (panel B). Effect of sildenafil (10⁻¹⁰-10⁻⁵ M) on the amplitude of EFS-evoked relaxation 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), and indomethacin (10⁻⁵ M), and indomethacin (10⁻⁵ M) (not shown). Data represent mean \pm sd of 8 experiments.

78x24mm (300 x 300 DPI)

For Review Only