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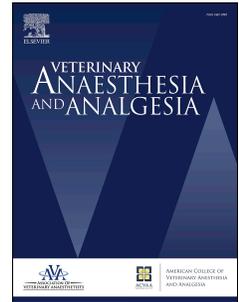
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**Evaluation of methadone concentrations in bitches and in umbilical cords after epidural or systemic administration for caesarean section. A randomized trial**

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**Running title:** methadone for caesarian section in dogs

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### **Authors' contributions**

All authors participated in data acquisition; moreover CL and AB performed data's analysis and interpretation, and drafted the paper; MC participated in data's interpretation and revised the paper; GB participated in data's analysis and in revising the paper; SA participated in data's interpretation and in drafting the paper; DZ participated in the study design, revised the paper and approved the final version; AZ participated in data interpretation and revised the paper; NR conceived the study, participated in the data's interpretation, revised the paper and approved the final version.

### **Declaration of interest**

Authors declare no conflict of interest

1 **Word count: 3899**

2 **Abstract**

3 **Objective** To measure plasma methadone concentrations in bitches and the umbilical  
4 cords of their puppies after systemic or epidural administration.

5 **Study design** Prospective, randomised, clinical study.

6 **Animals** A total of 27 healthy pregnant female dogs undergoing caesarean section, 4.3  
7  $\pm$  2.3 years of age and weighing  $19.9 \pm 13.2$  kg.

8 **Methods** The dogs were randomly divided into three groups: 1) intramuscular  
9 methadone ( $0.3 \text{ mg kg}^{-1}$ ) (group MET;  $n = 9$ ); 2) epidural methadone ( $0.1 \text{ mg kg}^{-1}$ )  
10 (group METEPI;  $n = 9$ ) and 3) epidural lidocaine ( $4.4 \text{ mg kg}^{-1}$ ) (group CON-control  
11 group;  $n = 9$ ). Ten minutes before induction, methadone was administered  
12 intramuscularly to the group MET dogs. Anaesthesia was induced with propofol and  
13 maintained with isoflurane. Cardiovascular and respiratory parameters were monitored  
14 throughout the anaesthesia. After induction, epidural anaesthesia was administered to  
15 dogs in groups METEPI and CON. Before any treatment (T0) and, as soon as the last  
16 foetus was removed from the uterus (T1), venous blood samples were collected from  
17 each dog using heparinised tubes; the umbilical cords were collected and stored at -  
18  $80^{\circ}\text{C}$  until pharmacological analysis was carried out. The samples were analysed using  
19 ultra performance liquid chromatography.

20 **Results** The cardiorespiratory parameters of the bitches and of the puppies at birth, and  
21 the Apgar scores did not differ significantly between groups. At T1 both the median  
22 maternal methadone plasma concentration and the median methadone umbilical cord

23 concentration were significantly higher in group MET compared to group METEPI  
24 ( $p=0.0018$  and  $p=0.004$ ). The maternal plasma concentration was significantly higher  
25 than the concentration in the umbilical cords ( $p=0.05$ ) in group METEPI but not in  
26 group MET ( $p=0.25$ ).

27 **Conclusions and clinical relevance** Epidural methadone ( $0.1 \text{ mg kg}^{-1}$ ) administered to  
28 bitches undergoing caesarean section is associated with lower umbilical cord  
29 concentrations as compared with intramuscularly administered methadone at higher  
30 dosages ( $0.3 \text{ mg kg}^{-1}$ ).

31

32 **Keywords** caesarean section, dog, epidural anaesthesia, methadone.

33

## 34 **Introduction**

35 In bitches, more than 60% of dystocias result in surgical caesarean section (Münnich &  
36 Kuchenmeister 2009; Smith 2012; Martins-Bessa et al. 2016). The anaesthetic protocol  
37 adopted for a caesarean section should provide adequate muscle relaxation, analgesia  
38 and narcosis to ensure optimal operating conditions (De Cramer et al. 2017). In  
39 addition, it must be safe for both the bitch and the foetus since most anaesthetic drugs  
40 cross the foetus blood-brain barrier, resulting in neurological and cardiorespiratory  
41 depression of the puppies (Luna et al. 2004; Conde Ruiz et al. 2016). Currently,  
42 propofol and isoflurane are the anaesthetic drugs most commonly used for the induction  
43 and maintenance of general anaesthesia for caesarean sections in dogs (Doebeli et al.  
44 2013). In a previous study, the authors found that the administration of propofol for the  
45 induction of general anaesthesia was associated with less depression of the puppies at  
46 birth and a lower mortality rate as compared with the administration of thiopentone, or  
47 ketamine and midazolam (Luna et al. 2004).

48 In human medicine, epidural anaesthesia (EA), combining local anaesthetic drugs and  
49 opioids, has been used to provide analgesia during labour or caesarean section for many  
50 years (Bader et al. 1995; Fernando et al. 1997; Jones et al. 2012). Luna et al. (2004)  
51 described that the respiratory rate of puppies born from bitches receiving EA with  
52 methadone or lidocaine was higher when compared with those born from bitches in  
53 which midazolam/ketamine or propofol for induction and enflurane for maintenance of  
54 general anaesthesia were used (Luna et al. 2004). Epidural anaesthesia, by means of a  
55 sparing effect or elimination of inhalant anaesthetics, decreases the risk of excessive  
56 neonatal respiratory depression, and improves the comfort of the bitch that is then more  
57 likely to nurse the puppies after delivery (Aarnes & Bednarski 2015; Robertson 2016).

58 However, when local anaesthetic are administered epidurally, hypotension is a common  
59 complication, due to a sympathetic blockade, especially in haemodynamic-  
60 compromised animals (Jones 2001).

61 Epidurally administered methadone did not induce significant cardiovascular alterations  
62 in healthy dogs (Campagnol et al. 2012). To date, no information is available regarding  
63 the placental transfer of methadone in dogs and the respective maternal/foetal plasma  
64 concentration ratios. The aim of this study was to compare methadone concentrations in  
65 the plasma of the bitches, and those in the veins and arteries of the umbilical cords of  
66 their puppies after systemic or epidural administration during caesarean section.

## 67 **Materials and Methods**

### 68 **Animals**

69 The study was conducted in accordance with the provisions of European Directive  
70 2010/63/UE, adopted by the Italian Government. Privately owned pregnant female dogs  
71 which were presented to the Veterinary Teaching Hospital (VTH) of the University of  
72 Bologna for dystocia and which underwent emergency caesarean section from December  
73 2014 to December 2016 were included in this study up to a maximum of 30 dogs in  
74 accordance with the Local Ethical Committee. The number of animals included in the  
75 study was based on the mean of number of dogs admitted for caesarean section at the  
76 VTH in the previous five years. Written informed consent was obtained from the owner  
77 of each dog before starting the procedure.

78 The health status of each bitch was assessed by means of clinical examination, and  
79 haematological and biochemical parameters. Upon arrival, each bitch underwent a  
80 complete obstetrical examination and an ultrasound evaluation.

81 Bitches under one year of age and those previously treated with methadone (7 days  
82 before anaesthesia for caesarean section) were excluded from the study.

### 83 *Study design*

84 All the bitches included were randomly divided into three groups using a random dice  
85 roll. Allocation into a specific group was carried out by the same anaesthetist using  
86 online software (<http://www.roll-dice-online.com>).

87 Epidural lidocaine 2% ( $4.4 \text{ mg kg}^{-1}$ ) was administered to the dogs in the control group  
88 (group CON), intramuscular (IM) methadone ( $0.3 \text{ mg kg}^{-1}$ ) to the dogs in group MET,  
89 and epidural methadone ( $0.1 \text{ mg kg}^{-1}$ ) to group METEPI. The same expert anaesthetist  
90 who was aware of treatment designation performed the anaesthesia and the epidural  
91 puncture.

### 92 *Anaesthetic protocol*

93 Ten minutes before induction, methadone (Eptadone; Molteni Farmaceutici, Italy) ( $0.3$   
94  $\text{mg kg}^{-1}$ ) was administered IM in the quadriceps muscles to the bitches in group MET  
95 while, in the dogs in the other two groups, an analogous volume of saline solution  
96 (NaCl 0.9%-placebo; BBraun, Germany) was administered using the same route. After  
97 IM administration, intravenous catheters (22 gauge) were placed into both the left and  
98 the right cephalic veins. The left cephalic vein was used for drug injection and for the  
99 administration of Lactated Ringer's solution (Ringer lattato; ACME, Italy) at the rate of  
100  $10 \text{ mL kg}^{-1} \text{ hour}^{-1}$ ; the right cephalic vein was used for blood collection.

101 Anaesthesia was induced with propofol (Propovet; Esteve, Italy), administered  
102 intravenously (IV), and titrated to effect to allow endotracheal intubation. Endotracheal

103 intubation was attempted when masticatory and eyelid muscle tone were decreased, and  
104 a ventromedial rotation of the eyeball was observed. General anaesthesia was  
105 maintained with isoflurane (Isoflo; Abbott Laboratories Ltd, IL, USA) delivered in  
106 oxygen (100%) via a rebreathing system; the vaporizer was adjusted by the anaesthetist  
107 in order to obtain a stable surgical anaesthetic depth based on physical signs (reflexes).

108 During the procedure, the following parameters were continuously monitored with a  
109 multiparametric monitor (Datex-Ohmeda- S3; Datex-Ohmeda Inc, WI, USA) and  
110 recorded every five minutes: heart rate (HR), respiratory rate ( $f_R$ ), end-tidal carbon  
111 dioxide tension ( $P_{E'}CO_2$ ), fraction of expired isoflurane in % ( $F_{E'}Iso$ ), haemoglobin  
112 oxygen saturation using a pulse oximeter ( $SpO_2$ ), non-invasive blood pressure using a  
113 Doppler device (Minidrop ES-100 VX; Hadeco, Japan) and body temperature using an  
114 oesophageal probe. A forced-air warming blanket (Bair Hugger; 3M, UK) was used to  
115 maintain physiologic body temperature. The duration of the anaesthesia was defined as  
116 the time from the anaesthetic induction to the extubation of the bitches.

117 After instrumentation, and five minutes after induction, the bitches in groups METEPI  
118 and CON were positioned in sternal recumbency with the hind limbs positioned forward  
119 in order to administer the epidural block into the lumbosacral space (L7-S1); the correct  
120 placement of the spinal needle (BD Spinal Needle; Becton Dickinson, Spain) was  
121 confirmed using the hanging drop technique. Epidural anaesthesia was administered  
122 using methadone or lidocaine 2% (Lidocaina cloridrato; S.A.L.F. Spa, Italy) in groups  
123 METEPI and CON, respectively. Before injection, the methadone and the lidocaine  
124 were both diluted with NaCl 0.9%, if needed, in order to achieve a final volume of 0.25  
125 mL kg<sup>-1</sup> up to a maximum of 10 mL. The epidural injection was administered slowly  
126 over one minute. Hypotension was defined as systolic blood pressure (SAP) lower than

127 80 mmHg. In the case of hypotension, isoflurane administration was decreased, if  
128 possible, and a bolus of crystalloid (10 mL kg<sup>-1</sup>) was administered intravenously. In the  
129 case of persistent hypotension (more than ten minutes), dobutamine was administered  
130 (0.005-0.01 mg kg<sup>-1</sup> minute<sup>-1</sup>).

131 Within 5 minutes of birth, HR using a stethoscope,  $f_R$  by inspection of the thorax and  
132 the Apgar score (modified for puppies by Veronesi et al. 2009) were evaluated and  
133 recorded. In detail, the Apgar score was applied to evaluate the vitality and distress of  
134 the newborns and ranged from 0 to 3 meaning severe distress; 4 to 6 meaning moderate  
135 distress and 7 to 10 meaning no distress. After the last puppy was taken from the uterus,  
136 methadone was administered IM once at a dose of 0.1 mg kg<sup>-1</sup> in the bitches in group  
137 CON for treating postoperative pain. All the bitches were discharged from the VTH  
138 soon after recovery. After discharge, postoperative pain was managed by the referral  
139 private veterinarian.

#### 140 *Sample collection*

141 Immediately before the IM administration of methadone (group MET) or a placebo  
142 solution (groups METEPI and CON) (T0) and as soon as the foetuses were removed  
143 from the uterus (T1), venous blood (2 mL) was collected from each bitch using  
144 heparinised tubes and was immediately centrifuged. The plasma was then stored at  
145 -80°C until the assay was carried out. Blood samples were collected from the right  
146 venous catheter; before each sampling, 2 mL of blood was collected and was then  
147 reinjected in order to avoid contamination with the flushing solution. After blood  
148 collection, the venous catheter was flushed with 2 mL of NaCl 0.9% saline solution.

149 As soon as the last puppy had been taken from the uterus, the umbilical cords were  
150 removed and stored individually in sterile vials at -80°C until analysis.

#### 151 *Sample analysis*

152 The plasma samples were extracted following a previously published method (Shakleya  
153 et al. 2007) with slight modifications. After thawing the samples at 4°C, 200 µL of  
154 plasma was transferred to a microtube, and deuterated internal standard (methadone-d3)  
155 was added, followed by 600 µL of acetonitrile. The tube was then vortex-mixed for 30  
156 seconds, centrifuged at 7'000 ×g at 4°C for 10 minutes; the supernatant was then  
157 evaporated to dryness under a gentle nitrogen stream at 35°C. The dry extract was  
158 finally reconstituted with 200 µL of mobile phase, consisting of a mixture of 0.1%  
159 formic acid in water and acetonitrile (80/20, v/v), and was vortex-mixed for 30 seconds  
160 before transferring the contents into a chromatography vial.

161 A procedure previously validated in humans (De Castro et al. 2013) was adapted to  
162 measure the methadone concentrations in the canine umbilical cords. After thawing the  
163 collected pools of umbilical cords at 4°C, for each brood 1 g was homogenized in a  
164 polypropylene tube containing 5 mL of water using a T25 digital Ultra-Turrax (IKA;  
165 Germany) at 24,000 rpm for 2 minutes. The internal standard and 50 µL of formic acid  
166 10% were then added; the tube was then vortex-mixed for 30 seconds and centrifuged  
167 for 15 minutes at 5'000 ×g at 4°C. The supernatant underwent a clean up step using an  
168 SPE Oasis MCX 3cc 60mg cartridge (Waters; Milford, MA, USA) and was eluted with  
169 3 mL of a methanol-ammonium hydroxide (95:5, v/v) solution. The sample was then  
170 evaporated to dryness under nitrogen and was reconstituted with 200 µL of a 0.1%  
171 formic acid aqueous solution-acetonitrile (80/20, v/v) mixture. After vortex-mixing and

172 centrifuging for 10 minutes at  $10'000 \times g$  at  $4^{\circ}\text{C}$ , 150  $\mu\text{L}$  of the sample was transferred  
173 into a chromatography vial for analysis.

174 Methadone quantification was carried out using a Waters Aquity ultra performance  
175 liquid chromatography (UPLC) binary pump equipped with an Aquity BEH C18 ( $50 \times$   
176  $2.1 \text{ mm}$ ,  $1.7 \mu\text{m}$ ) column and coupled to a Quattro Premier XE triple quadrupole mass  
177 spectrometer (Waters; Milford). The column was kept at  $35^{\circ}\text{C}$  and the mobile phase  
178 consisted of a mixture of 0.1% formic acid aqueous solution and acetonitrile at a 0.5 mL  
179  $\text{minute}^{-1}$  flow rate under programmed conditions. The mass spectrometer operated in  
180 positive electrospray ionisation (ESI+) and in MRM (multiple reaction monitoring)  
181 mode. The specific transitions observed were: methadone:  $310 > 265 \text{ m/z}$  and  $310 > 105$   
182  $\text{m/z}$  and methadone-D3:  $313.1 \rightarrow 268 \text{ m/z}$ . The capillary voltage was set at 2.00 kV, and  
183 the source and desolvation temperatures were 120 and  $350^{\circ}\text{C}$ , respectively; desolvation  
184 and cone gas flows were set at 700 and  $100 \text{ L hour}^{-1}$ , respectively.

185 The analytical method was validated in accordance with the  
186 EMEA/CHMP/EWP/192217/2009 guidelines before the experiment started, providing  
187 satisfying performances over a range of 0.5 to  $500 \text{ ng mL}^{-1}$ .

#### 188 *Statistical analysis*

189 Demographic data and plasma concentrations are reported as mean  $\pm$  standard deviation  
190 (SD). The 95% confidence interval (CI) of the median is reported for plasma and  
191 umbilical methadone concentrations. The data were evaluated for normality using a  
192 Shapiro-Wilk test. Normal data were compared using one-way ANOVA while not  
193 normally distributed data were compared using a Kruskal Wallis test. The plasma  
194 concentration of each bitch and that obtained from the respective umbilical cord pool

195 were compared using a Wilcoxon test for paired samples. The statistical data were  
196 calculated using commercial software (MedCalc 6.3; MedCalc Software, Belgium).  
197 Data were considered significant at  $p < 0.05$ . At the end of the study, a post hoc power  
198 calculation was carried out using computer software (STATA; StataCorp, TX, USA).

## 199 **Results**

### 200 *Animals*

201 Twenty-seven healthy bitches were included in the study, nine bitches in each group.  
202 The mean weight and ages of the bitches was  $25.3 \pm 13.6$  kg,  $19.2 \pm 12.2$  kg and  $15.3 \pm$   
203  $13.3$  kg, and  $3.8 \pm 1.6$  years,  $4.1 \pm 1.5$  years and  $4.8 \pm 3.3$  years for groups CON, MET  
204 and METEPI, respectively. No statistical differences in age and weight were detected  
205 among the three groups. All the bitches required an emergency caesarean section due to  
206 dystocia. The dogs included belonged to several breeds, with the French bulldog being  
207 the most represented. The mean anaesthesia duration did not differ significantly  
208 between groups and was  $87.7 \pm 24.8$  minutes in group CON,  $95.6 \pm 26.0$  minutes in  
209 group MET and  $80.0 \pm 32.1$  minutes in group METEPI. The number of puppies born in  
210 each group, their HRs, their  $f_{RS}$ , the Apgar scores and the mortality rates did not differ  
211 significantly between groups and are reported in Table 1.

212 The dose of propofol used for induction of general anaesthesia did not differ  
213 significantly between groups and was  $3.7 \pm 2.2$  mg kg<sup>-1</sup> for group MET,  $5.1 \pm 1.6$  mg  
214 kg<sup>-1</sup> for group METEPI and  $4.7 \pm 1.6$  mg kg<sup>-1</sup> for group CON

215 The mean FE<sub>Iso</sub> was  $1.4 \pm 0.2\%$  in group CON and  $1.3 \pm 0.2\%$  in both group MET and  
216 group METEPI without no significant differences between groups. In each dog that was  
217 administered an epidural, the aspiration of the “hanging drop” of saline from the needle

218 hub was observed and increased resistance was felt by the operator while advancing the  
219 needle through the *ligamentum flavum*.

220 In group MET, a preterm caesarean section was performed in a Springer spaniel in  
221 which labor began early (approximately 55 days of gestation) because of hypoluteidism  
222 and none of her three puppies responded to the resuscitation manoeuvre. In the same  
223 group, three puppies from a French bulldog died at birth. In group METEPI, one puppy  
224 from a pug and one puppy from an English setter died at birth, but foetal suffering had  
225 been diagnosed upon arrival.

226 In group MET, the last puppy was removed from the uterus and the second blood  
227 sample was collected from each bitch (T1) at  $35.1 \pm 9.9$  minutes after IM methadone  
228 administration; in group METEPI, the last puppy was removed  $25.5 \pm 11.9$  minutes  
229 after epidural administration and a blood sample (T1) was collected. The time interval  
230 between methadone administration and T1 did not differ significantly between the two  
231 methadone-treated groups.

232 For all bitches, recovery from anaesthesia was smooth and uneventful. The bitches did  
233 not have any complications related to the anaesthesia, to the epidural technique or to the  
234 surgical procedure.

#### 235 *Cardiovascular parameters*

236 The mean HR,  $f_R$  and SAP of the bitches are reported in Table S1. These parameters did  
237 not differ significantly between the groups at any time point.

238 Some dogs in group METEPI (6/9) experienced transient hypotension (SAP < 80  
239 mmHg) while only 3/9 and 4/9 of the dogs in groups MET and CON, respectively had

240 hypotension during the procedure. However, the incidence of episode of hypotension  
241 did not differ between groups. The transient hypotension was treated by decreasing the  
242 isoflurane and by administering a bolus of crystalloid (Lactated Ringer's solution 10  
243 mL kg<sup>-1</sup>). More dogs in group METEPI (3/6 hypotensive dogs) experienced mild and  
244 transient hypotension within five minutes after induction; among the hypotensive  
245 animals, the mean SAP was 69 ± 4 mmHg.

#### 246 *Plasma and umbilical cord concentrations*

247 In the samples collected from the animals in group CON, no signal corresponding to  
248 methadone was detected.

249 At T1, the median maternal methadone plasma concentration was 19.0 (range 9.0-56.2;  
250 95% CI: 13.5-31.3) ng mL<sup>-1</sup> and 6.4 (range 5.1-9.6; 95% CI: 5.2-8.8) ng mL<sup>-1</sup> in groups  
251 MET and METEPI, respectively (Figure 1). The median methadone concentration in the  
252 umbilical cords was 15.6 (range 12.1-25.3; 95% CI: 12.3-23.3) ng mL<sup>-1</sup> in group MET  
253 and 3.9 (range 1.2-8.4; 95% CI: 1.9-5.4) ng mL<sup>-1</sup> in group METEPI (Figure 1). Both the  
254 median methadone concentrations in the maternal plasma and in the umbilical cords  
255 were statistically higher in group MET compared to group METEPI ( $p=0.0018$  and  
256  $p=0.004$ , respectively). In group MET the maternal methadone plasma concentration  
257 and the umbilical cord concentration did not differ significantly ( $p=0.25$ ).

258 In group METEPI, the methadone concentration was higher in the maternal plasma  
259 compared to the concentration in the umbilical cord ( $p=0.046$ ) and they differed by  
260 39%.

#### 261 **Discussion**

262 In the present study, the methadone concentrations in the plasma of the bitches and in  
263 the umbilical cords of their puppies were evaluated after epidural or systemic  
264 administration for analgesia during emergency caesarean section.

265 There is a paucity of information concerning the pharmacokinetics of methadone in  
266 dogs after epidural administration (Garrett et al. 1985; Schmidt et al. 1994; Ingvast-  
267 Larsson et al. 2010). In human medicine, epidurally administered methadone reached  
268 peak plasma concentrations within 10-20 minutes, similar to those observed after IM  
269 injection, in the same patients (Max et al. 1985). In the present study, the blood samples  
270 for the determination of maternal methadone plasma concentration were collected  
271 approximately 9.6 minutes later in group METEPI but IM administered methadone  
272 resulted in higher maternal plasma and foetal umbilical cord concentrations compared  
273 with those obtained after epidural administration. This difference, despite the 10  
274 minutes of delay for blood collection, might not be only due to due to the different  
275 routes of administration but also to the different dosages used. In addition, the plasma  
276 methadone concentration after IM injection obtained at the moment in which the last  
277 puppies were removed from the uterus was wide: 9.0-56.2 ng mL<sup>-1</sup>. This is an  
278 expression of individual variability, as previously described after extravascular injection  
279 of methadone in dogs (Ingvast-Larsson et al. 2010). In fact, absorption after  
280 extravascular injection depends on several factors; in particular, on regional perfusion  
281 but also on the age, size and breed of the dogs considered (Kukanich & Wiese 2015).

282 In the present study, overall mortality among the puppies was 12%, slightly higher than  
283 that reported by Luna and colleagues (2004). Since only emergency caesarean sections  
284 were included in the present study, the mortality rate might have been influenced by  
285 several factors other than the analgesic drug administered, such as the conditions of

286 labour and the puppies' clinical condition before the anaesthetic procedure. Moreover,  
287 in all the dogs, propofol, which crosses the placenta quickly, was administered for  
288 anaesthesia induction, and general anaesthesia was maintained with isoflurane in all  
289 dogs. Conversely, in the study of Luna and colleagues (2004), dogs in which epidural  
290 anaesthesia was performed did not receive general anaesthesia, and the puppies born  
291 from those bitches experienced the least respiratory depression compared with those  
292 born from bitches receiving propofol, thiopentone or ketamine and midazolam for  
293 induction and with enflurane for maintenance of general anaesthesia. In humans,  
294 neonatal depression after propofol administration for anaesthesia induction is correlated  
295 with the dose administered (Sanchez-Alcaraz et al. 1998). However, the authors did not  
296 evaluate the correlation between the dosage of propofol used and the outcome of the  
297 puppies as this was beyond the aim of the study.

298 The present study has several limitations. First, only twenty-seven dogs were included;  
299 they all underwent emergency caesarean section, and foetal sufferance had already been  
300 diagnosed at presentation. Therefore, morbidity and mortality among the puppies cannot  
301 be correlated only with the anaesthetic protocol used. A scheduled caesarean section  
302 could have led to different results in the outcomes of the puppies.

303 In addition, dogs are multiparous and have short umbilical cords; the technique applied  
304 allowed evaluation of the umbilical cord concentration using a pool of samples without  
305 differentiating among the puppies, or between venous and arterial umbilical samples  
306 (Desprats et al. 1991). In humans, the evaluation of the concentration of the drugs in the  
307 umbilical cord is of interest for evaluating the correlation between the anaesthetic  
308 protocol and the outcome of the foetus.

309 The cardiorespiratory parameters of the bitches were similar in the three groups. The  
310 limited number of animals included might account for the lack of statistically significant  
311 differences in cardiorespiratory parameters between groups; however the primary aim of  
312 the study was evaluation of the methadone concentration rather than the physiological  
313 effect of the anaesthetic protocols used. Hypotension after epidural administration of  
314 local anaesthetics is mainly seen in sick animals in which the compensatory  
315 mechanisms are unable to counteract the reduced sympathetic tone (Jones 2001). In  
316 healthy animals, epidural lidocaine or epidural methadone have been reported to  
317 produce only minimal cardiorespiratory changes (Cruz et al. 1997). When methadone  
318 was administered epidurally in isoflurane anaesthetised dogs at dosages of  $0.1 \text{ mg kg}^{-1}$ ,  
319 a gradual increase in HR and SAP was observed; however, these changes were not  
320 significant when compared with placebo-treated dogs (Bosmans et al. 2011). When the  
321 effects of methadone ( $0.5 \text{ mg kg}^{-1}$ ) administered by an epidural or an intravenous route  
322 were compared, no significant differences in HR and blood pressure were reported  
323 (Campagnol et al. 2012). In pregnant animals, blood pressure monitoring is pivotal, and  
324 hypotension must be promptly corrected. When pregnant animals are positioned in  
325 dorsal recumbency, the enlarged uterus can compress the caudal vena cava thus  
326 reducing the venous return to the heart chambers and consequently, the cardiac output;  
327 therefore, decreased uterine perfusion may result. In the present study the blood  
328 pressure was measured non-invasively; however, the doppler device was demonstrated  
329 to have a specificity of 97% and a sensitivity of 56% in detecting hypotension in  
330 anaesthetised dogs (Kennedy & Barletta 2015). Even if some animals experienced  
331 hypotension, it was immediately and successfully corrected by decreasing the isoflurane

332 and administering a bolus of fluids. Moreover, most of the dogs in which methadone  
333 was administered by the epidural route experienced hypotension soon after induction.

334 In group METEPI, no premedicant drugs were administered prior to induction and a  
335 higher dose of propofol was necessary to achieve an adequate anaesthetic plane to  
336 perform intubation. Interestingly, both methadone treated groups had a similar mean  
337  $FE_{Iso}$ . This was in accordance with a previous experimental study regarding isoflurane  
338 anaesthetised dogs which showed a similar sparing effect of epidural and intravenous  
339 methadone up to 2.5 hours after administration, with the epidural methadone providing  
340 a longer lasting sparing effect (Campagnol et al. 2012).

341 Another limitation is the fact that no intraoperative and postoperative pain evaluation  
342 was carried out and the correct execution of the epidural anaesthesia was not confirmed  
343 by means of a radiographic evaluation. All the epidural punctures were performed by  
344 the same expert anaesthetist and their success was confirmed in all dogs in groups  
345 METEPI and CON by the hanging drop technique, namely by the operator who felt the  
346 change in resistance while passing the *ligamentum flavum* and inspected the needle hub  
347 for signs of cerebrospinal fluid or blood before drug injection. In addition, the  
348 anaesthetic plane was stable in all patients and no changes in HR,  $f_R$  and SAP were  
349 observed in response to the surgical stimulation. The hanging drop technique has been  
350 described to be an effective method of confirming needle tip location in the extradural  
351 space in 88% of dogs in which EA was performed in sternal recumbency (Naganobu &  
352 Hagio 2007). Failures of the technique were described to be only false negative results;  
353 on the contrary, false positive responses were not observed.

354 **Conclusion**

355 In conclusion, epidurally administered methadone ( $0.1 \text{ mg kg}^{-1}$ ) in bitches undergoing  
356 caesarean section was associated with lower umbilical cord methadone concentrations  
357 as compared to concentrations after IM methadone administration at higher dosages ( $0.3$   
358  $\text{mg kg}^{-1}$ ). These protocols applied for emergency caesarean section were associated with  
359 a puppy mortality rate of 17.7%. More studies are needed to determine the effects of  
360 these protocols on the clinical parameters of puppies born from scheduled caesarean  
361 sections.

362

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442

443 **Figure 1** Box-and-whisker plots of the methadone concentrations in the maternal  
 444 plasma and the umbilical cords of puppies born from 18 bitches undergoing caesarean  
 445 section. Blood samples from the bitches and the umbilical cords were collected as soon  
 446 as the last puppy was removed from the uterus. The bitches received systemic  
 447 methadone (group MET) or epidural methadone (group METEPI). (  $\bar{\quad}$  ) Maternal  
 448 plasmatic concentration; (  $\text{----}$  ) umbilical cord concentration. (\*) statistically  
 449 significant difference ( $p < 0.05$ ).

450 **Table 1** Number of puppies, heart rate (HR), respiratory rate ( $f_R$ ), Apgar score and  
 451 mortality in puppies born from bitches undergoing emergency caesarean section and  
 452 receiving epidural lidocaine 2%, 4.4 mg kg<sup>-1</sup> (group CON), intramuscular methadone,  
 453 0.3 mg kg<sup>-1</sup> (group MET) or epidural methadone, 0.1 mg kg<sup>-1</sup> (group METEPI). Heart  
 454 rate,  $f_R$  and Apgar score were recorded within 5 minutes after birth. Heart rate and  $f_R$  are  
 455 reported as means  $\pm$  standard deviation; Apgar scores are reported as median (range).

456

| Parameters                            | group CON        | group MET        | group METEPI  |
|---------------------------------------|------------------|------------------|---------------|
| Number of puppies                     | 35               | 35               | 30            |
| HR (beats minute <sup>-1</sup> )      | 193.7 $\pm$ 37.7 | 165.3 $\pm$ 79.1 | 171 $\pm$ 70  |
| $f_R$ (breaths minute <sup>-1</sup> ) | 10.1 $\pm$ 4.1   | 9.1 $\pm$ 5.2    | 9.2 $\pm$ 5.2 |

|                           |          |          |          |
|---------------------------|----------|----------|----------|
| Apgar score               | 7 (0-10) | 6 (0-10) | 6 (0-10) |
| Mortality of puppies (n=) | 2        | 6        | 4        |
| Mortality rate (%)        | 5.7      | 17.1     | 13.3     |

457

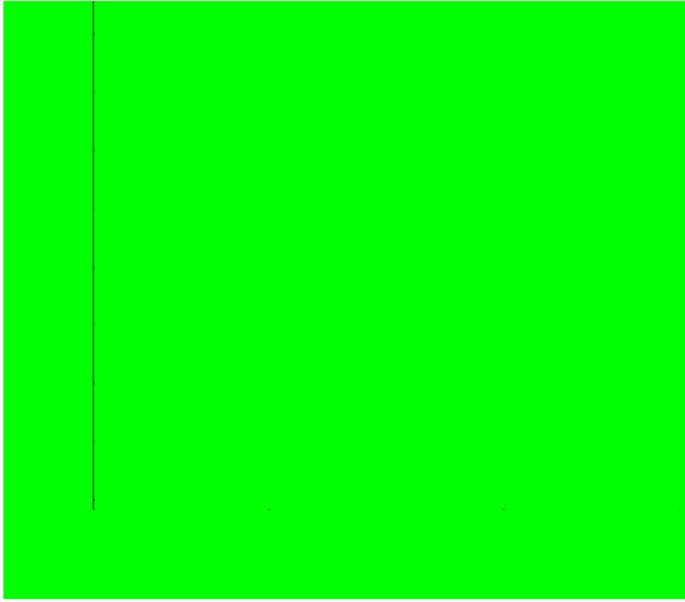
458 **Table S1** - Systolic blood pressure (SAP), heart rate (HR), respiratory rate ( $f_R$ ) of 27  
459 bitches undergoing emergency caesarean section and receiving epidural lidocaine  
460 (group CON), intramuscular methadone (group MET) or epidural methadone (group  
461 METEPI) are reported. Data were collected every five minutes from induction until the  
462 end of the anaesthesia and are reported as mean  $\pm$  SD.

| Minutes | SAP (mmHg)        |                  |                  | HR (beats minutes <sup>-1</sup> ) |                  |                  | $f_R$ (breaths minutes <sup>-1</sup> ) |                 |                 |
|---------|-------------------|------------------|------------------|-----------------------------------|------------------|------------------|----------------------------------------|-----------------|-----------------|
|         | CON               | MET              | METEPI           | CON                               | MET              | METEPI           | CON                                    | MET             | METEPI          |
| 5       | 135.4 $\pm$ 20.2  | 122.4 $\pm$ 26.6 | 104.0 $\pm$ 29   | 126.1 $\pm$ 20.7                  | 132.6 $\pm$ 8.8  | 128.0 $\pm$ 19.8 | 17.1 $\pm$ 6.1                         | 18.7 $\pm$ 12.6 | 25.2 $\pm$ 14.5 |
| 10      | 132.6 $\pm$ 22.5  | 129.6 $\pm$ 16.4 | 110.2 $\pm$ 22.3 | 126.4 $\pm$ 20.5                  | 128.3 $\pm$ 12.4 | 128.3 $\pm$ 15.6 | 17.6 $\pm$ 7.8                         | 20.1 $\pm$ 11.7 | 23.9 $\pm$ 13.0 |
| 15      | 126.1 $\pm$ 18.9  | 129.0 $\pm$ 16.3 | 113.6 $\pm$ 17.0 | 122.9 $\pm$ 29.2                  | 121.8 $\pm$ 13.7 | 121.7 $\pm$ 9.5  | 15.9 $\pm$ 9.0                         | 19.9 $\pm$ 11.7 | 20.6 $\pm$ 14.7 |
| 20      | 118.5 $\pm$ 25.5  | 118.2 $\pm$ 20   | 107.1 $\pm$ 14.0 | 122.1 $\pm$ 26                    | 120.3 $\pm$ 14.5 | 120.6 $\pm$ 16.4 | 15.1 $\pm$ 6.0                         | 18.8 $\pm$ 10.4 | 15.9 $\pm$ 5.8  |
| 25      | 116.1 $\pm$ 30.6  | 116.1 $\pm$ 21.6 | 107.2 $\pm$ 14.1 | 117.7 $\pm$ 22.9                  | 119.0 $\pm$ 15.5 | 118.1 $\pm$ 16.0 | 13.1 $\pm$ 4.1                         | 18.8 $\pm$ 10.4 | 16.2 $\pm$ 11.0 |
| 30      | 125.1 $\pm$ 25.8  | 120.9 $\pm$ 20.1 | 105.7 $\pm$ 16.7 | 116.4 $\pm$ 21.7                  | 115.7 $\pm$ 17.8 | 119.9 $\pm$ 17.1 | 13.4 $\pm$ 6.7                         | 19.1 $\pm$ 10.7 | 14.1 $\pm$ 7.2  |
| 35      | 103 $\pm$ 36.5    | 116.0 $\pm$ 17.5 | 105.6 $\pm$ 17.0 | 121.5 $\pm$ 16.4                  | 114.7 $\pm$ 13.8 | 117.8 $\pm$ 18.8 | 15.6 $\pm$ 7.4                         | 19.2 $\pm$ 10.5 | 15.5 $\pm$ 7.2  |
| 40      | 115.1 $\pm$ 40.4  | 118.0 $\pm$ 20.1 | 110.7 $\pm$ 20.1 | 122.8 $\pm$ 13.7                  | 117.1 $\pm$ 14.3 | 116.4 $\pm$ 15.7 | 16.3 $\pm$ 8.2                         | 18.4 $\pm$ 10.7 | 15.1 $\pm$ 9.0  |
| 45      | 121.5 $\pm$ 22.8  | 117.0 $\pm$ 15.9 | 104.7 $\pm$ 18.0 | 124.4 $\pm$ 15.5                  | 115.6 $\pm$ 18.7 | 117.0 $\pm$ 17.3 | 18.9 $\pm$ 8.9                         | 17.4 $\pm$ 10.8 | 15.6 $\pm$ 7.7  |
| 50      | 114.7 $\pm$ 24.5  | 114.4 $\pm$ 18.6 | 105.4 $\pm$ 14.2 | 120.5 $\pm$ 14.0                  | 113.0 $\pm$ 17   | 117.4 $\pm$ 19.5 | 14.5 $\pm$ 6.8                         | 19.2 $\pm$ 11.6 | 17.1 $\pm$ 7.2  |
| 55      | 123.8 $\pm$ 28.4  | 115.8 $\pm$ 22.3 | 104.5 $\pm$ 15.7 | 124.4 $\pm$ 12.0                  | 115.2 $\pm$ 15.7 | 118.4 $\pm$ 20.2 | 16.3 $\pm$ 7.5                         | 17.8 $\pm$ 11.2 | 17.0 $\pm$ 8.0  |
| 60      | 118.0 $\pm$ 20.4  | 118.8 $\pm$ 21.2 | 107.8 $\pm$ 18.8 | 121.3 $\pm$ 10.7                  | 114.1 $\pm$ 16.4 | 115.4 $\pm$ 20.6 | 15.3 $\pm$ 8.7                         | 17.0 $\pm$ 10.5 | 17.8 $\pm$ 8.0  |
| 65      | 113.5 $\pm$ 36.7  | 121.3 $\pm$ 20.1 | 113.8 $\pm$ 19.0 | 118.4 $\pm$ 7.2                   | 110.6 $\pm$ 15.6 | 113.8 $\pm$ 26.9 | 15.0 $\pm$ 7.5                         | 18.7 $\pm$ 10.1 | 12.5 $\pm$ 7.0  |
| 70      | 108.6 $\pm$ 25.8  | 121.5 $\pm$ 15.4 | 104.5 $\pm$ 12.7 | 118.6 $\pm$ 7.8                   | 110.1 $\pm$ 17.8 | 115.5 $\pm$ 28.8 | 13.6 $\pm$ 7.9                         | 18.7 $\pm$ 10.1 | 13.5 $\pm$ 7.0  |
| 75      | 113.7 $\pm$ 22.8  | 121.6 $\pm$ 10.8 | 113.0 $\pm$ 11.6 | 118.7 $\pm$ 8.0                   | 112.6 $\pm$ 18   | 112.5 $\pm$ 25.0 | 16.4 $\pm$ 7.8                         | 19.0 $\pm$ 10.1 | 14.0 $\pm$ 7.0  |
| 80      | 108.2 $\pm$ 24.8  | 125.6 $\pm$ 15.6 | 110.0 $\pm$ 12.0 | 115.8 $\pm$ 8.0                   | 112.4 $\pm$ 17.0 | 125.0 $\pm$ 8.7  | 16.3 $\pm$ 7.0                         | 19.0 $\pm$ 10.1 | 14.0 $\pm$ 7.0  |
| 85      | 110.0 $\pm$ 25.4  | 122.5 $\pm$ 13.0 | 107.3 $\pm$ 7.0  | 113.3 $\pm$ 10.3                  | 110.0 $\pm$ 19.0 | 126.7 $\pm$ 11.5 | 17.8 $\pm$ 6.9                         | 20.2 $\pm$ 12.0 | 10.7 $\pm$ 5.1  |
| 90      | 123.0 $\pm$ 30.40 | 122.1 $\pm$ 9.8  | 111.3 $\pm$ 6.1  | 112.5 $\pm$ 8.7                   | 110.0 $\pm$ 19   | 126.7 $\pm$ 11.5 | 14.0 $\pm$ 5.6                         | 19.8 $\pm$ 12.0 | 13.7 $\pm$ 5.1  |
| 95      |                   | 121.5 $\pm$ 9.4  | 116.3 $\pm$ 11.5 | 114.3 $\pm$ 10.0                  | 108.4            | 129.3 $\pm$ 16.2 | 11.3 $\pm$ 2.3                         | 19.8 $\pm$ 12.0 | 14.3 $\pm$ 6.0  |
| 100     |                   | 121.2 $\pm$ 11.4 | 110.7 $\pm$ 4.6  | 108.5 $\pm$ 9.1                   | 110.4            | 130.0 $\pm$ 17.3 | 11.0 $\pm$ 1.4                         | 19.8 $\pm$ 12.0 | 12.7 $\pm$ 4.0  |

|     |                 |                  |                 |                  |                  |               |                 |                |
|-----|-----------------|------------------|-----------------|------------------|------------------|---------------|-----------------|----------------|
| 105 | $115.0 \pm 7.0$ | $108.3 \pm 12.3$ | $108.5 \pm 9.2$ | $118.0 \pm 10.6$ | $130.0 \pm 14.1$ | $8.5 \pm 4.9$ | $22.8 \pm 11.6$ | $11.5 \pm 9.1$ |
| 110 | $115.5 \pm 6.4$ | $118.0 \pm 8.5$  |                 | $117.5 \pm 10.4$ | $126.5 \pm 12.0$ |               | $22.8 \pm 11.6$ | $10.5 \pm 7.8$ |
| 115 | $115.0 \pm 7.4$ | $127.0 \pm 4.2$  |                 | $117.0 \pm 10.3$ | $127.5 \pm 17.7$ |               | $22.8 \pm 11.6$ | $10.5 \pm 7.8$ |
| 120 | $115.0 \pm 6.1$ | $114.5 \pm 7.8$  |                 | $117.5 \pm 10.4$ | $130.0 \pm 21.1$ |               | $22.3 \pm 11.9$ | $9.5 \pm 6.4$  |
| 125 |                 | $112.0 \pm 5.6$  |                 |                  | $127.5 \pm 17.7$ |               |                 | $10.0 \pm 7.1$ |
| 130 |                 | $119.5 \pm 6.4$  |                 |                  | $131.5 \pm 23.3$ |               |                 | $11.5 \pm 9.2$ |

463

ACCEPTED MANUSCRIPT



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