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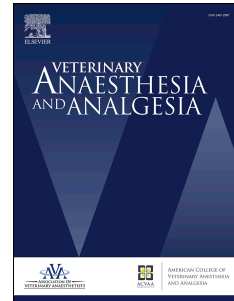
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Evaluation of methadone concentrations in bitches and in umbilical cords after epidural or systemic administration for caesarean section. A randomized trial

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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 ± 13.2 kg.

8 **Methods** The dogs were randomly divided into three groups: 1) intramuscular
9 methadone (0.3 mg kg^{-1}) (group MET; $n = 9$); 2) epidural methadone (0.1 mg kg^{-1})
10 (group METEPI; $n = 9$) and 3) epidural lidocaine (4.4 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°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 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 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 mg kg^{-1}) was administered to the dogs in the control group
88 (group CON), intramuscular (IM) methadone (0.3 mg kg^{-1}) to the dogs in group MET,
89 and epidural methadone (0.1 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 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⁻¹ 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⁻¹) was administered intravenously. In the
129 case of persistent hypotension (more than ten minutes), dobutamine was administered
130 (0.005-0.01 mg kg⁻¹ minute⁻¹).

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⁻¹ 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°C , 150 μL 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 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°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 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 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°C , respectively; desolvation
184 and cone gas flows were set at 700 and 100 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 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 ± 13.6 kg, 19.2 ± 12.2 kg and $15.3 \pm$
203 13.3 kg, and 3.8 ± 1.6 years, 4.1 ± 1.5 years and 4.8 ± 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 ± 24.8 minutes in group CON, 95.6 ± 26.0 minutes in
209 group MET and 80.0 ± 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 ± 2.2 mg kg^{-1} for group MET, 5.1 ± 1.6 mg
214 kg^{-1} for group METEPI and 4.7 ± 1.6 mg kg^{-1} for group CON

215 The mean $\text{FE}'\text{Iso}$ 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 ± 9.9 minutes after IM methadone
228 administration; in group METEPI, the last puppy was removed 25.5 ± 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⁻¹). 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⁻¹ and 6.4 (range 5.1-9.6; 95% CI: 5.2-8.8) ng mL⁻¹ 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⁻¹ in group MET
253 and 3.9 (range 1.2-8.4; 95% CI: 1.9-5.4) ng mL⁻¹ 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⁻¹. 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 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 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 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 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). () Maternal
 448 plasmatic concentration; (----) 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⁻¹ (group CON), intramuscular methadone,
 453 0.3 mg kg⁻¹ (group MET) or epidural methadone, 0.1 mg kg⁻¹ (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 ⁻¹)	193.7 \pm 37.7	165.3 \pm 79.1	171 \pm 70
f_R (breaths minute ⁻¹)	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

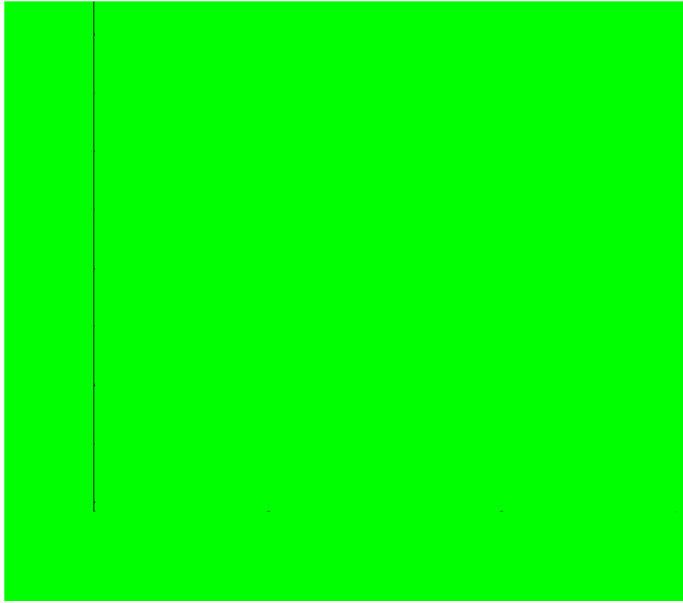
458 **Table S1** - Systolic blood pressure (SAP), heart rate (HR), respiratory rate (f_R) of 27
459 bitches undergoing emergency caesarean section and receiving epidural lidocaine
460 (group CON), intramuscular methadone (group MET) or epidural methadone (group
461 METEPI) are reported. Data were collected 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 ⁻¹)			f_R (breaths minutes ⁻¹)		
	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 ± 7.0	108.3 ± 12.3	108.5 ± 9.2	118.0 ± 10.6	130.0 ± 14.1	8.5 ± 4.9	22.8 ± 11.6	11.5 ± 9.1
110	115.5 ± 6.4	118.0 ± 8.5		117.5 ± 10.4	126.5 ± 12.0		22.8 ± 11.6	10.5 ± 7.8
115	115.0 ± 7.4	127.0 ± 4.2		117.0 ± 10.3	127.5 ± 17.7		22.8 ± 11.6	10.5 ± 7.8
120	115.0 ± 6.1	114.5 ± 7.8		117.5 ± 10.4	130.0 ± 21.1		22.3 ± 11.9	9.5 ± 6.4
125		112.0 ± 5.6			127.5 ± 17.7			10.0 ± 7.1
130		119.5 ± 6.4			131.5 ± 23.3			11.5 ± 9.2

463

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