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The effect of uterine lavage on soluble CD14 (sCD14), chemokine ligand 2 (CCL2) and interleukin (IL)-10 levels in mares with postpartum metritis

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Summary

Post-partum metritis in mares is a life-threatening condition associated with severe clinical signs due to endotoxemia, and it is often followed by complications such as laminitis. Repeated large volume uterine lavages are commonly recommended as a part of the treatment protocol in order to remove endotoxin-laden contents from the uterus. It has, however, also been suggested that lavages may increase the uptake of endotoxin into the circulation, leading to a deterioration of clinical signs. Endotoxemia is associated with release of a multitude of inflammatory mediators regulating the immune response. The aim of this study was to evaluate if uterine lavage influences serum levels of the inflammation markers soluble CD14 (sCD14), chemokine (C-C motif) ligand 2 (CCL2) and interleukin (IL)-10 in mares with postpartum metritis. Serum samples were collected from eight mares treated for metritis at a university teaching hospital. Mares with fever, tachycardia, and/or leukopenia, and haemosanguineous or purulent intrauterine fluid within one week of foaling were included

in the study. Serum samples were taken before uterine lavage, and 15 and 30 min after starting the lavage. The concentrations of sCD14, CCL2 and IL-10 were determined with a fluorescent bead-based immunoassay. There were no significant differences between sCD14, CCL2 or IL-10 levels at the different sampling times. Heart rate was significantly lower after uterine lavage than before. The differences in body temperature and leukocyte count before and after lavage were not significant. In conclusion, uterine lavage had no immediate effect on the serum concentration of sCD14, CCL2 or IL-10, nor did it affect clinical parameters negatively.

Keywords: equine, postpartum metritis, soluble CD14 sCD14), chemokine ligand 2 (CCL2), interleukin-10 (IL-10), retained fetal membranes

1. Introduction

Post-partum metritis in mares is a life-threatening condition associated with severe clinical signs due to endotoxemia, and it is often followed by complications such as laminitis. Metritis is a common sequelae of retained fetal membranes (RFM) and other complications associated with pregnancy and parturition, such as dystocia [1,2]. With severe metritis, inflammation of the uterine wall permits bacteria and toxins to enter the systemic circulation, leading to septicemia, endotoxemia and laminitis. Mares with toxic metritis become febrile and depressed, with an elevated heart rate and injected mucous membranes [1,3,4].

Treatment of mares with RFM and toxic metritis include frequently administered uterine ecbolics, such as oxytocin, to prevent fluid and debris accumulation in the uterus, broad-spectrum antimicrobial therapy, and non-steroidal anti-inflammatory drugs. Bacterial culture typically yields mixed growth with both gram-positive and gram-negative components, and

49 antimicrobial treatment often consists of a combination of β-lactams and aminoglycosides, or
50 oral potentiated sulfonamides. Non-steroidal anti-inflammatory drugs are also used because
51 of their anti-inflammatory, analgesic and antiendotoxic effects [2].
52
53 Repeated large volume uterine lavage is often recommended as a part of the treatment
54 protocol. The benefits of uterine lavage in the treatment of uterine infections have been
55 summarized by Brinsko [5] and Canisso et al. [2], and include reduction of bacterial load by
56 removing exudate and debris from the uterine lumen, physical clearance of uterine contents
57 by stimulation of uterine contractions, and recruitment of neutrophils by inducing transient
58 irritation of the endometrium. However, it has also been suggested that lavages could
59 increase blood flow, inflammation and vascular permeability locally. This could increase the
60 uptake of endotoxins and bacterial translocation into the circulation, leading to an
61 exacerbation of endotoxemia and a deterioration of clinical systemic signs [2,6]. To date,
62 there is no scientific evidence available supporting this statement.
63
64 Endotoxins, the lipopolysaccharide (LPS) outer wall of gram-negative bacteria, can
65 accumulate in the infected contents of a postpartum uterus. If LPS is absorbed into the
66 systemic circulation, it causes clinical signs of endotoxemia through activation of pro- and
67 anti-inflammatory processes regulated by various cytokines and chemokines [7,8].
68 Intravenous infusion of LPS in horses can function as a model for studying the inflammatory
69 cascade and causes similar clinical signs as in naturally occurring endotoxemia [9]. There is a
70 multitude of inflammatory mediators regulating the immune response. Soluble CD14 (sCD14)
71 is a component of the innate immune system that reduces adverse effects of bacterial LPS
72 with subsequent anti-inflammatory effects [10], and circulating concentrations of sCD14

73 increase during acute and chronic inflammation [11]. It is an important inflammatory marker
74 in a variety of diseases in humans, with increased values associated with poor outcome [12-
75 15]. Soluble CD14 has been evaluated as a biomarker in neonatal foals with septicemia and
76 horses with recurrent airway obstruction, and foals with septicemia had significantly higher
77 sCD14 concentrations in their circulation than healthy foals [10]. As a clinical diagnostic tool,
78 sCD14 is superior to directly evaluating LPS levels [16].
79
80 In a study on healthy horses, LPS infusion resulted in an increase in plasma concentrations of
81 chemokines and interleukins, such as chemokine (C-C motif) ligand 2 (CCL2) (previously
82 known as MCP-1) and interleukin -10 (IL-10), but not an increase in sCD14 [17]. Interleukin-
83 10 has a pivotal role in the control and downregulation of inflammation [18,19], and it has
84 been demonstrated that IL-10 levels increase rapidly in response to LPS infusion in mice
85 [19,20]. In horses, infusion with LPS increases mRNA expression for IL-10 in peripheral
86 leukocytes [21,22] and plasma concentrations of IL-10 in horses [23].
87
88 The aim of this study was to measure serum concentrations of the inflammatory markers
89 sCD14, CCL2, and Il-10 in mares with postpartum metritis, and to evaluate if uterine lavage
90 increases the levels of these markers.
91

92 **2. Material and methods**

93

94 **2.1. Horses**

95 Samples were collected at 13 uterine lavages performed on eight mares (seven Warmblood
96 mares, one Finnhorse; ages 4-22 years) at the University Animal Hospital, University of

97 Helsinki, Finland. Mares were included into the study if large volume uterine lavages were a
98 part of their treatment for postpartum metritis at the hospital, and they met at least two of the
99 following criteria within one week of parturition: leukopenia (WBC < 5.0 x 10^9 cells/L),
100 elevated body temperature (T > 38.2°C), elevated heart rate (HR > 44 bpm), retained fetal
101 membranes for more than three hours after parturition, or accumulation of
102 haemosanguineous or purulent fluid in the uterus. Ethical approval was not required for this
103 study according to the Finnish Act on the Protection of Animals Used for Scientific or
104 Educational Purposes (497/2013).
105
106 **2.2. Uterine lavage**
107 The uterine lavage technique used was the one widely used in practice, and has been
108 described by Brinsko [5]. After cleaning the perineum with running water and a povidone
109 iodine scrub, a sterile nasogastric tube was inserted through the cervix, and tap water
110 (containing a small amount of povidone-iodine, and in some cases also table salt to achieve a
111 0.9% NaCl solution) was instilled into the uterus by gravity flow. Fluid was added until the
112 uterine lumen was filled with fluid, and then siphoned out. The volume of fluid was not
113 measured, and varied according to the size of the uterus. Lavage was continued until the
114 returning fluid was relatively clear, and repeated one to three times a day, depending on the
115 color and turbidity of the uterine fluid, and the clinical status of the mare. Sedation with
116 detomidine and butorphanol was used in 3/13 lavages. Oxytocin was given (20 IU im) in
117 association with the uterine lavage in all cases.
118
119 **2.3. Sample collection**

120 A 10-ml blood sample was collected for serum analysis at the start of each uterine lavage
121 (T0), and at 15 min (T1) and 30 min (T2) after the initial sample. The blood samples were
122 allowed to clot for 30 minutes at 20°C, separated by centrifugation at 1000 g for 10 minutes
123 and then stored at −70°C until analyzed. Two samples at T1, and two samples at T2 are
124 missing from the data, as these samples were not collected.
125
126 **2.4. Clinical data**
127 The clinical data were obtained and recorded by the treating veterinarians at the hospital, with
128 repeated physical examinations and treatment according to the needs of each case, and the
129 time intervals of the examinations and uterine lavages varied. The mares were admitted into
130 veterinary care at 1.4 ± 0.6 (mean ± SEM) days after parturition, and the first uterine lavage
131 with collection of blood samples for the study was done at 2.0 ± 0.4 days after parturition. On
132 admission, the mean body temperature of the mares was 38.1 ± 0.2°C and the mean heart
133 rate was 47.1 ± 3.2 bpm. The duration of hospitalization was 4.3 ± 0.7 days. All of the mares
134 presented with a large amount of uterine fluid with varying appearance, and 3/8 mares had
135 vulvar discharge.
136
137 All mares were treated with antimicrobials (trimethoprim sulfadiazine or a combination of
138 penicillin and gentamycin), flunixin meglumine and oxytocin. Complications associated with
139 parturition and the postpartum period included RFM (8/8 mares), dystocia (7/8 mares),
140 abortion (2/8 mares), colic (1/8 mares) and laminitis (1/8 mares). All of mares survived to
141 discharge.
142
143 **2.5. Soluble CD14, CCL2 and IL-10 assays**

144 The cytokines were quantified using species-specific fluorescent bead-based immunoassays
145 as described previously [10,17,24]. Monoclonal antibodies against recombinant equine
146 cytokines were coupled to fluorescent beads (anti-equine IL-10 clone 492-228 coupled to
147 bead 34; anti-equine CD14 clone 105 coupled to bead 38; anti-CCL2 coupled to bead 37).
148 Beads coupled with antibodies were mixed and diluted in buffer (final concentration of 10^5
149 beads/mL each), and added to each well of the microtiter plate. Cell culture supernatants
150 containing recombinant cytokine/IgG-fusion proteins were used as standards for
151 quantification. Serum samples were added and incubated for 30 min at room temperature.
152 Biotinylated anti-cytokine detection antibodies were then added: antiequine IL-10 clone 165–
153 228, anti-CCL2, and anti-CD14 mAb clone 59, followed by streptavidin–phycoerythrin
154 (Invitrogen, USA). The assay was analyzed in a Luminex 200 instrument (Luminex Corp.,
155 USA) and the data were reported as median fluorescence intensities. The cytokine
156 concentrations in samples were calculated according to the logistic 5p formula ($y = a + b/(1 +$
157 $(x/c)^d)^f$) (Luminex 100 Integrated System 2.3.). The multiplex intra- and inter-assay variability
158 has been described elsewhere [10,24]. The detection threshold was 1 ng/ml for sCD14, 1
159 pg/ml for CCL2, and >15 pg/ml for IL-10.
160
161 **2.6. Data analysis**
162 The arithmetic mean and standard error of mean were used for descriptive statistics. Data
163 sets were tested for normality using a Kolmogorov-Smirnov test. The values of sCD14, CCL2
164 and IL-10 were non-normally distributed. Nonparametric Friedman tests for related samples
165 were used on commercial statistical software (IBM SPSS Statistics, USA) to analyze the
166 differences in concentrations in sCD14, CCL2 and IL-10. The Wilcoxon signed ranks test was
167 used for the pairwise comparisons of clinical data from before and after uterine lavage, and

168 between the first and second lavages. Spearman's rank correlation was used to analyze the
169 relationship between the concentrations of sCD14, CCL2, and IL-10 at time point T0 and
170 mare age, number of days from parturition to admission, body temperature, heart rate and
171 WBC count on admission and at T0, time interval from NSAID administration to sample
172 collection, and duration of treatment. The results are reported as mean ± SEM. The
173 significance level was set at 0.05.
174
175 **3. Results**
176
177 **3.1. Soluble CD14, CCL2 and IL-10**
178 The concentrations of sCD14, CCL2 and IL-10 at the different time points after uterine lavage
179 are shown in Table 1, with the results of individual mares shown in Figures 1, 2 and 3.
180
181 Interleukin-10 was undetectable in 16/35 samples from 5/8 mares. In order to assess whether
182 mares with undetectable levels of IL-10 were less severely affected than mares with
183 detectable levels, the concentrations of sCD14 and CCL2 and clinical data were compared in
184 these two groups of mares. The concentrations of sCD14 (2671 ± 268.1 vs. 2894.6 ± 533.2
185 ng/ml) and CCL2 (357.1 ± 114.6 vs. 605.0 ± 205.4) did not differ between the two mare
186 groups. There were no significant differences between the two mare groups in age, body
187 temperature on admission, body temperature, heart rate and WBC count before or after
188 uterine lavage, or duration of treatment.
189
190 The time interval from NSAID administration to sample collection was 8.9 ± 2.4 h, with a
191 significant positive correlation between this time interval and the concentration of CCL2 at T0

192 (r = 0.85, p = 0.004), and the concentration of CCL2 at T1 (r = 0.74, p = 0.037). The time
193 interval from NSAID administration to sample collection was not correlated to the
194 concentrations of sCD14, CCL2 at T2, or IL-10.
195
196 Samples were collected during two consecutive lavages in 5/8 mares. The concentrations of
197 sCD14, CCL2 and IL-10 at the end of the first lavage and at the start of the second lavage are
198 shown in Table 2.
199
200 The concentration of IL-10 at T0 was positively correlated to mare age (r = 0.71, p = 0.048).
201 The correlations between sCD14, CCL2, and IL-10 at T0 and number of days from parturition
202 to admission, retained fetal membranes, body temperature, heart rate and WBC count on
203 admission and at T0, and duration of treatment were not significant.
204

205 3.2. Clinical data

206 Body temperature, heart rate and white blood cell count at the time of uterine lavage and after
207 lavage are shown in Table 3.
208

209 4. Discussion

210 There were no significant differences between the levels of sCD14, CCL2 or IL-10 at the
211 different sampling times, indicating that uterine lavage did not cause any immediate changes
212 in the production or release of these components in the mare. It may be argued that collection
213 of serum samples at 15 and 30 min after the start of the uterine lavage may have been too
214 early for any significant change in cytokine concentrations. There were, however, no
215 significant differences between concentrations of sCD14, CCL2 and IL-10 at the end of the

216 first lavage as compared to the start of the second lavage either, with a time interval of 10 -
217 23.5 hours. In dogs that received an LPS-infusion, the levels of CCL2 and IL-10 increased
218 within one to four hours, and CCL2 levels were still elevated at 24 h, compared to placebo-
219 treated dogs [25]. A similar timeline, with changes in IL-10 levels being evident at two hours
220 after LPS-infusion, has been observed in mice [19]. Bonelli et al. [17] reported that the levels
221 of sCD14 did not change significantly after LPS infusion when samples were collected hourly
222 for three hours and again at 24 h, but the horses in their study had higher pre-infusion levels
223 of sCD14 than reported in healthy horses elsewhere [16,26]. The rise in IL-10 seems to occur
224 faster in horses than in human, with peak values observed 1 hr after LPS infusion [17].
225
226 The mean levels of sCD14 in the mares in our study were tenfold higher than the levels
227 Wagner et al. [10] reported in healthy foals and adult horses, which depicts the level of
228 systemic inflammation in our mares. The levels of sCD14 in our study were similar to those
229 reported in sick horses hospitalized due to colic or other acute endotoxemia-related disease
230 (2.48 ± 1.99 µg/ml) [26]. In contrast, the levels of sCD14 in the study by Silva et al. [26] were
231 of the same order of magnitude as those reported by Bonelli et al. [17] in healthy horses
232 before and after LPS infusion. The mean levels of CCL2 in our study were markedly lower
233 than the post-LPS infusion values in the study by Bonelli et al. [17], where the authors
234 reported a significant increase in CCL2 compared to values before LPS infusion. This
235 supports the notion that CCL2 should have increased in our study if there had been an
236 increase in LPS in the systemic circulation of the mare. However, the mentioned studies have
237 been performed on low numbers of horses, and the serum cytokine concentrations need to be
238 evaluated in a larger number of horses of different breed, age and gender to establish normal
239 cytokine concentrations in horses.

240

241 If uterine lavage would cause an exacerbation of acute endotoxemia, a detectable and
242 significant increase in IL-10 would be expected [17], but this was not observed in our study,
243 with serum levels of IL-10 being undetectable in 16/35 samples of 5/8 mares. Plasma
244 concentrations of IL-10 have been shown to increase rapidly in adult horses after LPS
245 infusion, with concentrations peaking at 2 h and returning to values indistinguishable from
246 those in the control group by 6 h [23]. According to their clinical signs, the mares with
247 undetectable IL-10 were not less severely affected than the mares with detectable levels in
248 our study on a limited number of animals. It is unclear why the levels of IL-10 were so low in
249 the studied mares, but it may be because of relatively mild disease and early intervention in
250 these cases. Despite the results of Pusterla et al. [27] and Gold et al. [28] showing that IL-10
251 gene expression could be used as a prognosticator of survival in neonatal septic foals, serum
252 levels of IL-10 did not differ between septic foals and healthy control foals when samples
253 were collected at the time of enrolment, and at 24 and 48 h of treatment [29]. In
254 experimentally induced bacterial placentitis in mares, IL-10 concentrations in maternal or fetal
255 serum were not affected four to six days after inoculation, compared to controls [30]. The
256 concentration of IL-10 may not be a reliable marker for endotoxemia, but the timing of sample
257 collection may also be critical if the levels of IL-10 change rapidly. The concentration of IL-10
258 at T0 was correlated to mare age, similarly to a previous study by Schnabel et al. [31], but
259 mares with undetectable levels did not differ in age compared to mares with detectable levels.
260 This could be related to the small sample size in our study.

261

262 The time interval from NSAID administration to sampling was correlated to concentrations of
263 CCL2 at T0 and T1. Higher values of CCL2 were associated with a longer time interval from

264 medication, possibly reflecting the decrease in the anti-inflammatory effects of the drug with
265 time. Heart rate was significantly lower after uterine lavage, when evaluated at 6.4 ± 0.9 h
266 after the start of the lavage, than before the procedure. The differences in body temperature
267 and leukocyte count before and after lavage were not significant. Heart rate at T0 was in most
268 cases measured while the mare was in stocks being prepared for lavage, which may have
269 elevated the heart rate in some cases. Heart rate after lavage was measured in the mare's
270 stall, where the mare was likely more calm. Uterine lavage was not the only treatment
271 modality that could have affected these variables, as these horses were also treated with
272 antimicrobials, non-steroidal anti-inflammatories and oxytocin.
273
274 The samples for this study were collected within the first few days after parturition. Recently, it
275 was shown that mRNA expression of CCL2 and other chemokines is increased in the
276 allantochorion and endometrium of mares with retained fetal membranes [32], but the
277 concentrations of sCD14, CCL2 or IL-10 in the systemic circulation of periparturient mares are
278 not known. In our study, the levels of sCD14, CCL2, and IL-10 at T0 were not correlated to
279 the number of days from parturition to admission or whether or not the fetal membranes were
280 retained. However, it must be kept in mind that the sample size is limited. Research on post-
281 partum levels of inflammatory factors in other species show that in moderate-yielding cows,
282 gene expression of CCL2 in neutrophils was downregulated, while gene expression of IL-10
283 was upregulated, on the day of calving compared to one week before or after calving [33]. In
284 sows, levels of tumor necrosis factor (TNF)-α, IL-6, and serum amyloid A (SAA) increased at
285 the time of parturition and were still elevated at the last sampling time (36 hrs postpartum) in a
286 study by [34].
287

Uterine lavage is widely recommended in the literature [1,5,36] but an international practitioner survey [35] indicates that the use of large volume lavage in the initial treatment of RFM iss less common than one would expect. In the survey, large volume uterine lavage or the Burns' technique (filling the chorioallantoic sac with fluid) was used in the initial treatment of RFM by only 25% of respondents. More than half of these respondents who used these techniques preferred large volume lavage over the Burns technique. In cases in which the initial treatment for RFM failed, 60% of respondents reported using large volume lavage as part of continued treatment of the mare [35]. According to the results of our study, uterine lavage does not have a negative effect on the inflammatory status of the mare, and it can be used safely to remove infected and endotoxin-laden material from the uterus. In normal foalings and healthy postparturient mares, uterine lavage does not significantly affect uterine involution or uterine fluid accumulation evaluated at Day 11 postpartum [37], nor does it increase pregnancy rates [38,39] despite a reduction in endometrial polymorphonuclear cells at first and second postpartum estrus [39].

5. Conclusions

In conclusion, uterine lavage had no immediate effect on the serum concentration of sCD14, CCL2 or IL-10, nor did it affect clinical parameters. A decrease in heart rate was observed after uterine lavage, but this is likely due to a combined effect of all treatment modalities used.

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Conflict of interest

None to declare.

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455
456 **Table 1.**
457 Mean ± SEM of serum sCD14, CC2 and IL-10 concentrations for each blood-sampling time
458 (before (T0), 15 min after (T1), and 30 min after (T2) uterine lavage). N = 9 - 13.

	Sampling time			
	T0	T1	T2	
sCD14 (ng/ml)	2757.4 ± 251.5	2661.5 ± 249.7	2572.3 ± 170.3	NS (p=0.717)
CCL2 (pg/ml)	452.5 ± 106.5	456.0 ± 116.7	462.5 ± 120.6	NS (p=0.368)
IL-10 (pg/ml)	83.2 ± 37.5	61.0 ± 41.6	85.2 ± 46.3	NS (p=0.241)

459
460 **Table 2.**
461 Mean ± SEM of serum sCD14, CCL2 and IL-10 concentrations at the end of the first uterine
462 lavage and at the start of the second uterine lavage. The mean (± SEM) time interval between
463 the two lavages was 18.9 ± 2.4 hours. N=5.

	First lavage	Second lavage	
sCD14 (ng/ml)	2479.0 ± 182.2	3170.4 ± 428.9	NS (p=0.138)
CCL2 (pg/ml)	642.2 ± 199.7	275.8 ± 73.0	NS (p=0.138)
IL-10 (pg/ml)	143.4 ± 91.2	62.4 ± 44.2	NS (p=0.144)

464

465

466 **Table 3.**

467 Mean ± SEM of body temperature (BT), white blood cell count (WBC) and heart rate (HR) at

468 the time of uterine lavage, after lavage, and the time interval between measurements. N = 13.

	At lavage	After lavage	Time interval (hours)
BT (°C)	37.8 ± 0.13[a]	37.7 ± 0.08[a]	7.8 ± 1.6
WBC (10^9 cells/L)	7.2 ± 1.6[a]	6.0 ± 0.9[a]	40.5 ± 12.3
HR (bpm)	45.3 ± 2.4[a]	38.1 ± 3.9[b]	6.4 ± 0.9

469 [a,b]Different superscripts within the same row indicate a significant difference ($p<0.05$).