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Circulating and endometrial cell oxidative stress in dairy cows diagnosed with metritis

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1 **Use of Creatine kinase as marker for endometritis and infertility in beef cattle.**

2 **A. Ricci^a, S. Gallo^{ab}, P. Banchi^{a*}, M Santhia^b, A. Starvaggi Cucuzza^a, L. Vincenti^a**

3 ^a Dpt. Veterinary Science, University of Torino, Italy

4 ^b Large Animal Practitioner, Piemonte, Italy

5 *corresponding author: penelope.banchi@unito.it

6

7 **ABSTRACT**

8 In beef cows, a complete uterine involution requires about 30 days post-partum (pp) and a total
9 resumed estrous cycles is expected within 50 days pp, but uterine pathologies can delay these
10 processes, causing economic damage. In general, uterine pathologies delay the partum to
11 conception of 30 to 50 days than healthy cows. In double muscles breeds, uterine pathologies are
12 still present. Creatine kinase (CK) serum concentrations have been investigated in dairy cows as a
13 marker for endometritis, showing different values between healthy and diseased cows. The first
14 objective of this study is to define the basal CK serum concentrations for healthy beef cows, the
15 second consists in the evaluation of the accuracy of CK serum concentrations in detecting clinical
16 endometritis. Sixteen pregnant Piedmontese beef cows were used to determine the basal serum CK
17 concentration. Furthermore, another group of 264 non-pregnant Piedmontese cows were used to
18 assess CK performances as a diagnostic tool for clinical endometritis.

19 Healthy cows didn't show different concentration of CK mean than RB cows (216 ± 186 vs 268 ± 191
20 U/L, $P>0.05$) and PREG cows (189 ± 135 U/L $P>0.05$); whereas Endometritis 449 ± 263 showed a
21 significant higher CK mean of 449 ± 263 U/L ($P=0.0001$). In non-pregnant cows, 77% (203/264)
22 were healthy (HEALTHY) without any disease and 12.5% (33/264) were classified as repeat
23 breeding (RB). The total percentage of cows negative for endometritis was 89.4% (236/264),
24 whereas 10.6% (28/264) of the examined cows was diagnosed with clinical endometritis.

25 The PC for diseased cows resulted higher than healthy ones (144 ± 30 vs 87 ± 40 dpp; $P=0.006$) but
26 shorter than the PC of repeat breeder cows (191 ± 65 dpp; $P=0.003$). The same was for number of AI
27 per pregnancy. Diseased cows show higher number of insemination than healthy ones (3.1 ± 0.8 vs
28 1.9 ± 1.2), but not than RB cows, that shows 5.2 ± 1.3 insemination per pregnancy (Table 1).

29 The CK mean cut-off to predict endometritis from ROC curve was 241 U/L, showing good
30 accuracy (Se 92%, Sp 69%, AUC 0.81). Furthermore, CK wasn't accurate for infertility at 120, 150
31 days pp. This study underlines the potentiality of CK as a marker for endometritis. This could lead
32 to a preventive and not invasive on-field diagnostic method which could be implemented in the
33 health check routine of postpartum cows.

34

35 **Key words:** Creatine kinase, Piedmontese cow, Endometritis

36

37 **1. INTRODUCTION**

38 Beef cattle breeding is much less standardized than that of dairy cattle, in fact there are many
39 different breeds and crossbreed and farming systems, ranging from intensive to extensive [1].
40 Although the characteristics of some breeds are little investigated, the mistakes and low

41 reproductive performances are often caused by failure of information about nutritional
42 requirements, breeding and farming management. Current knowledge allows us to state that in beef
43 cows, a complete uterine involution requires about 30 days post-partum (pp) and a total resumed
44 estrous cycles is expected within 50 days pp [1]. Uterine pathologies can delay these processes,
45 causing economic damage to the farm. Piedmontese beef cow is a high-quality double-muscled
46 breed, due to a mutation of the myostatin gene [2] causing a muscular hypertrophy. Even if genetic
47 selection is trying to contain this phenomenon, Piedmontese cows are affected by a higher rate of
48 difficult delivery and dystocia with subsequent lower fertility [3, 4]. In our experience early and
49 non-invasive diagnosis of uterine pathologies is a key point to reduce partum to conception days
50 (PC), in order to decrease the number of inseminations per pregnancy and improve reproduction
51 performances.

52 Clinical endometritis is a common inflammatory condition of the uterus associated with bacterial
53 infection with purulent or muco-purulent uterine discharge with no systemic signs from 21 days
54 after calving [5]. It affects around 15-35% of cows at 4-6 weeks postpartum [6, 7] and it has severe
55 effects on fertility, causing poor reproductive performances with relevant consequences such as
56 reduction in pregnancy rate, increased time to conception and increased culling rate [6, 8].

57 Inflammation of the genital tract is a common condition in dairy and beef cows, but not all of the
58 cows affected by uterine contamination post-partum will develop uterine diseases.

59 Assessment of uterine discharge through vaginoscopy, manual examination of the vagina, or
60 Metricheck is the main diagnostic tool for endometritis [9]. Transrectal palpation of the uterus has
61 lower predictive value for the reproductive performances of the animal [5, 10]. Uterine cytology
62 performed by uterine lavage or cytobrush and endometrial biopsy are considered more reliable and
63 accurate diagnostic techniques [8, 9] but they are more invasive and not easy to perform on field.
64 The presence of vaginal exudate is referred as 'purulent vaginal discharge' (PVD) and it is
65 generally assumed that PVD is the result of endometritis, cervicitis/vaginitis or the combination of
66 both [11, 12].

67 The detrimental effects of endometritis and cervicitis/vaginitis on reproductive performance are
68 additive [13]. In general, cows affected with PVD need about 30 days more to become pregnant
69 than unaffected cows [6, 11, 14].

70 Beef cows lack the interference of milk production. Therefore, they have a simpler post-partum
71 management than dairy cows and a generally better fertility. Although, in double muscles breeds,
72 uterine pathologies are still present [15].

73 Acute phase proteins (APPs) are a very large family of inflammatory mediators and are considered
74 as markers for general acute response, such as inflammation, tissue damage and infection [16, 17].

75 Furthermore, APPs have been proposed to be markers for stress in cattle and other species [18-22].
76 Specifically, haptoglobin has been suggested to serve as indicator of endometritis [23]. However,
77 the use of such diagnostic biomarker is still controversial [3, 24].
78 Creatine kinase (CK) serum concentrations have been investigated as a marker for endometritis,
79 showing different values between healthy and diseased cows [25, 26]. CK is an intracellular
80 cytosolic enzyme that catalyzes the reaction of creatine and adenosine triphosphate (ATP) to
81 phosphocreatine and adenosine diphosphate (ADP) [27]. It is a dimeric molecule composed of two
82 subunits (M and B). Combinations of these subunits form the isoenzymes CK-MM, CK-MB, and
83 CK-BB. CK is abundant in tissues with elevated energy transfer such as skeletal muscle,
84 myocardium, and brain. In other visceral tissues [28], noticeable CK concentrations can be found in
85 the uterine tissue and in every inner organ [25]. The serum of healthy cows contains almost entirely
86 CK-MM, while inner organs contain mostly CK-BB. Mechanical and metabolic stress of the uterine
87 tissue is known to cause elevated CK activities before and after normal parturition in cows [29].
88 Furthermore, serum concentrations of CK 3 days after parturition are lower in healthy Holstein
89 cows (median of 121 U/l) than in cows with retained placenta (median 175 U/l), dystocia (median
90 310 U/l), milk fever (median of 385 U/l) [2], and abomasal displacement. [25]. However, elevated
91 CK serum concentrations can be expected whenever recumbency occurs, due to the neuromuscular
92 damage [30]. Weber et al. (2019) pointed out that recumbent Holstein cows show higher CK serum
93 concentrations than healthy ones at day 5 after parturition (mean of 5011.28 ± 13386.53 vs $666.44 \pm$
94 1645.44) [31]. As for endometritis, CK has been assessed in dairy cows [25] and in Iraqi buffalo
95 cows [24]; results showed that animals with endometritis had higher CK activity than healthy ones.
96 However, higher CK blood concentration were found in estrous beef cows than in non-estrous ones
97 [32].
98 To the best of our knowledge, CK has never been investigated as a diagnostic tool for endometritis
99 in beef cows. The first objective of this study is to define the basal CK serum concentrations of
100 healthy Piedmontese beef cows, the second consists in the evaluation of the accuracy of CK serum
101 concentrations in detecting clinical endometritis.

102

103 **2. MATERIAL AND METHODS**

104 **2.1 Animals enrollment**

105 The present study was carried out in two farms of similar size (approximately 100 breeding cows)
106 with similar management and nutrition. All animals were vaccinated for bovine viral diarrhea
107 (BVD) and infectious bovine rhinotracheitis (IBR); all farms were officially free from tuberculosis

108 and brucellosis. The cows were housed in free stalls with free access to food and water.
109 Sixteen Piedmontese beef cows >100 days-pregnant (PREG), that were used to determine the basal
110 serum concentration for CK in Piedmontese cows out of the post-partum period. Furthermore,
111 another group of 264 non-pregnant Piedmontese cows were used to assess CK performances as a
112 diagnostic tool for clinical endometritis.

113 Two-hundred and three (203/264) cows belonging to the latter group were deemed as healthy
114 (HEALTHY), 33 cows (33/264) required a number of artificial insemination (AI) higher than 3,
115 without presenting any uterine pathologies and were defined as repeat breeding cows (RB), whereas
116 another group included 28 (28/264) cows diagnosed with clinical endometritis (ENDO). These
117 cows were examined at 30±5 days post-partum and sorted into the HEALTHY or ENDO group
118 according to the result of the physical examination, which was always performed by the same
119 veterinarian.

120 Vaginal discharge was categorized as described by Williams et al. (2005), using a 4-point
121 classification system: 0 = no or clear mucus, 1 = mucus containing few flecks, 2 = discharge
122 containing less than 50% pus, 3 = discharge containing more than 50% pus. A blood sample was
123 collected from each animal during the clinical examination. All cows were submitted to AI based
124 on heat detection at 60±5 days postpartum.

125 **2.2 Blood samples collection and biochemical analysis**

126 Blood samples were collected by venipuncture from the coccygeal vein using an 8 ml evacuated
127 serum collection tube and a 20 G needle (Vacutainer® Venoject, Terumo, Leuven, Belgium); the
128 samples were immediately refrigerated and transported to the laboratory within 4 hours. The blood
129 was centrifuged at 2,000 rpm for 10 minutes and the serum was separate and stored at -20°C in 1 ml
130 SafeLock tubes (Eppendorf®, Hamburg, Germany).

131 CK was measured with a clinical chemistry analyzer KUADRO® BPC (Biosed s.r.l, Rimini, Italy)
132 with Creatine Kinase immunologic kinetic UV-test (MTD Diagnostics, Caserta, Italy) in accord
133 with International Federation of Clinical Chemistry (IFCC).

134 **2.3 Statistical analysis**

135 A simple descriptive statistical analysis was performed to calculate the CK mean and ds for PREG
136 cows to set the basal serum concentration for CK in Piedmontese beef cows.

137 Afterwards, HEALTHY (including RB) and ENDO cows were analyzed with a one-way ANOVA
138 statistical method between healthyRB (HEALTHY + RB) and diseased (ENDO) animals and also
139 by each status (HEALTHY, RB, ENDO) to point-out any difference in CK serum concentrations.
140 Furthermore, a one-way ANOVA statistical method was used to evaluate reproductive
141 performances such as partum-to-conception interval (PC) and number of AI among groups.
142 Bonferroni pot-hoc test was used for pairwise comparison.
143 A receiver operating characteristic (ROC) curve model (pROC) and the area under the curve
144 (cvAUC) were calculated to find the optimal CK cut-off point for evaluating clinical endometritis at
145 30 days pp and infertility (PC at 120 and 150 days and number of AI).
146 Data were indicated as mean \pm ds. P values \leq 0.05 were considered significant, and trends were
147 considered to be present at P values between 0.06 and 0.08. Statistical analyses were performed
148 using R statistical software (ver. 2.15.2).

149 **3. RESULTS**

150 Statistical analysis on the 16 pregnant cows (PREG) showed a mean CK concentration of 189 ± 135
151 U/L. As shown in *Table 1*, Healthy cows didn't show different concentration of CK mean than RB
152 cows PREG cows (216 ± 186 vs 268 ± 191 U/L vs 189 ± 135 U/L $P>0.05$) and in general HealthyRB
153 (233 ± 239 U/L, $P>0.005$); whereas Endometritis 449 ± 263 showed a significant higher CK mean of
154 449 ± 263 U/L ($P=0.0001$).

155 In non-pregnant cows, 77% (203/264) was healthy (HEALTHY) without any disease and 12.5%
156 (33/264) was classified as repeat breeding (RB) after three IA. Therefore, the total percentage of
157 cows negative for endometritis (healthyRB) was 89.4% (236/264), whereas 10.6% (28/264) of the
158 examined cows was diagnosed with clinical endometritis.

159 The PC of cows with endometritis resulted higher than healthy cows (144 ± 30 vs 87 ± 40 dpp;
160 $P=0.006$) but shorter than the PC of RB cows (191 ± 65 dpp; $P=0.003$); this applies to the number of
161 AI per pregnancy too, as endometritis cows show higher number of insemination than healthy ones
162 (3.1 ± 0.8 vs 1.9 ± 1.2), but not than RB cows, that shows 5.2 insemination per pregnancy (*Table 1*).

163 As showed in *Figure 1*, the ROC curve indicates a cut-off of 241 U/L for CK to predict
164 endometritis, showing good accuracy (Se 92%, Sp 69%, AUC 0.81). According to results showed in
165 *Table 2*, CK cannot be used as marker of infertility at 120, 150 days pp.

166 **4. DISCUSSION**

167 The aims of this study were to determine a CK range in healthy Piedmontese cows out of the post-
168 partum period and to investigate the CK as a marker for uterine pathologies.

169 To define CK concentration range in healthy cows, animals >100 days pregnant were selected, in
170 order to be out of the post-partum period that could influence CK serum concentrations and to avoid
171 the influence of the estrus, that it was showed to be associated with higher mean CK serum
172 concentrations by Crane *et al.* (2016).

173 PVD has been indicated detrimental on the reproductive performances of dairy cows with
174 percentage around 30% at 4-6 weeks postpartum [6], Although, very little information has been
175 reported about beef cows. Our group has previously demonstrated that Sub-Clinical Endometritis
176 (SCE) causes a 40-days delay in conception, compared to healthy cows [15] In the present study,
177 11% (28/264) of cows showed clinical endometritis. This is slightly lower percentage than the 15-
178 35% reported in dairy cows at 30 days [6, 7, 12], but no precise data about uterine disease in beef
179 cows are present in literature. It can be speculated that beef cows are not affected by a remarkable
180 metabolic imbalance and immunosuppression during the first postpartum and the transition period.
181 Therefore, beef cows are expected to show a lower incidence of uterine pathologies than dairy
182 cows.

183 Various acute phase proteins have been used in dairy and beef cows and in other species as
184 inflammatory and stress response markers but are not accurate markers for uterine disease. As
185 matter of fact, haptoglobin increases during the third week postpartum regardless of the health
186 status of the cow [33, 34]. Furthermore, it increases in many stress situations and clinical conditions
187 other than in uterine pathologies [35]. In accordance to other authors [24, 25], in our study CK
188 concentrations increase more in cows with uterine pathologies than in healthy and repeat breeding
189 cows. It is noticeable that although Piedmontese cows is a double muscle breed, CTRL and healthy
190 cows did not show any higher CK, and the basal CK concentration in of this study did not differ
191 from literature of dairy cows [24].

192 According to literature, 52.7% of RB cows showed to be positive to SCE [36]. In our study no
193 further cytology has been carried out to investigate the presence of SCE in RB cows, but all cows
194 that showed infertility (increased PC and number of AI per pregnancy) have been considered as RB.
195 Furthermore, since no data about CK values for SCE are available and RB cows in our study did not
196 show CK differences form healthy ones, we speculated that SCE does not influence the CK
197 concentration in beef cows.

198 No data about blood CK concentration in beef cows are available in literature, therefore a ROC
199 curve was used, and a cut-off value of 241 U/L was set as a reference for a precise diagnosis of
200 uterine pathology in postpartum, because of the high specificity and the good AUC.

201 The sensitivity of a test (also called the true positive rate) is defined as the proportion of individuals
202 with the disease who will have a positive result. Therefore, a highly sensitive test can be useful for

203 ruling out a disease if an individual has a negative result [37]. A highly specific test can be useful
204 for ruling in patients who have a certain disease. Unfortunately, this use of CK has some
205 limitations, since an external laboratory is necessary to process the samples, delaying the diagnosis
206 of at least 24-48 hours.

207

208 **5. CONCLUSION**

209

210 The results of this study underline the potentiality of CK as a marker for uterine disease, with the
211 final goal to use CK as a good and fast method for the diagnosis of uterine pathologies. This could
212 lead to a preventive and not invasive on-field diagnostic method which could be implemented in the
213 health check routine of postpartum cows. Further study should be carried out to better analyze the
214 best CK cut-off values also in dairy cows and to implement a quick tool to measure CK in order to
215 use it as a diagnostic marker for uterine pathologies on field.

216

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220

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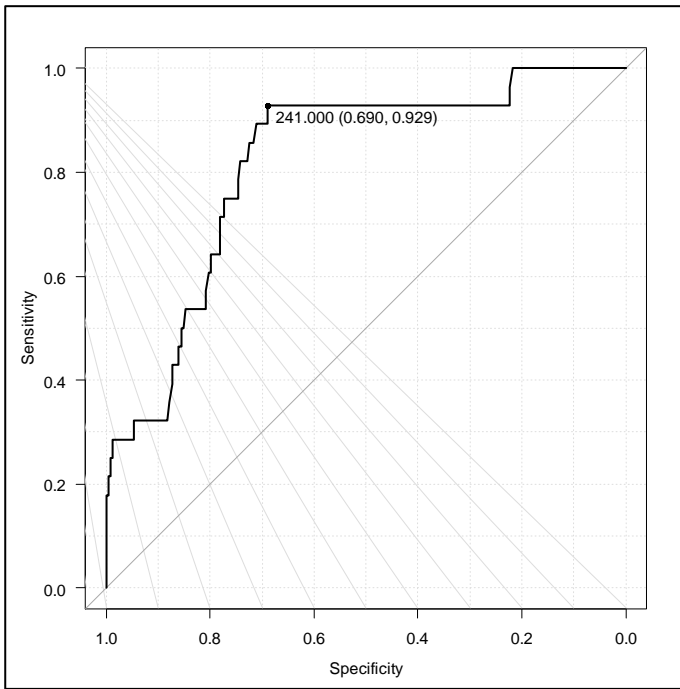
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Fig 1. ROC curve indicates a cut-off of 241 U/L for CK to predict endometritis. Sensitivity 92%, Specificity 69% and AUC 0.81.

341 **Table 1**

342 Serum CK concentration for healthy and pathological cows

343

	CK				PC			n AI/preg		
	N°	Mean	SD	P value	Mean	SD	P value	Mean	SD	P value
Healthy	203	216	186	0,0001	87	40	0,0006	1,9	1,2	0,002
Repeat breeders	33	268	191		191	65		5,2	0	
Endometritis	28	449	263		144	30		3,1	0,8	
HealthyRB	236	223	139	0,001	101	45	0,0003			
Endometritis	28	449	263		145	30				

344 **Healthy:** not diseased cows, **Repeat breeders:** cows without clinical uterine disease with >3 AI after parturition,

345 **HealthyRB** (Healthy cows + Repeat breeders), **Endometritis:** cows positive for endometritis using a 4-point

346 classification system: 0 = no or clear mucus, 1 = mucus containing few flecks, 2 = discharge containing less than 50%

347 pus, 3 = discharge containing more than 50% pus.

348

349 **Table 2**

350 Receiver operating characteristic curve results for Endometritis (Endo) and fertility (PC at 120 and

351 150 dpp).

352

	CK	Sp%	Se%	AUC	IC
Endo	241	69	92	0,81	0,73-0,89
Pc120	286	77	42	0,57	0,49-0,55
Pc150	341	82	34	0,59	0,47-0,65

353 **Endo:** Endometritis, **Pc120:** Partum to conception at 120 dpp, **Pc150:** partum to conception 150 dpp.

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