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

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

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ABSTRACT

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

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

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

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

Key words: Creatine kinase, Piedmontese cow, Endometritis

1. INTRODUCTION

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

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

Clinical endometritis is a common inflammatory condition of the uterus associated with bacterial infection with purulent or muco-purulent uterine discharge with no systemic signs from 21 days after calving [5]. It affects around 15-35% of cows at 4-6 weeks postpartum [6, 7] and it has severe effects on fertility, causing poor reproductive performances with relevant consequences such as reduction in pregnancy rate, increased time to conception and increased culling rate [6, 8]. Inflammation of the genital tract is a common condition in dairy and beef cows, but not all of the cows affected by uterine contamination post-partum will develop uterine diseases.

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

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

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

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

Furthermore, APPs have been proposed to be markers for stress in cattle and other species [18-22]. Specifically, haptoglobin has been suggested to serve as indicator of endometritis [23]. However, the use of such diagnostic biomarker is still controversial [3, 24].

Creatine kinase (CK) serum concentrations have been investigated as a marker for endometritis, showing different values between healthy and diseased cows [25, 26]. CK is an intracellular cytosolic enzyme that catalyzes the reaction of creatine and adenosine triphosphate (ATP) to phosphocreatine and adenosine diphosphate (ADP) [27]. It is a dimeric molecule composed of two subunits (M and B). Combinations of these subunits form the isoenzymes CK–MM, CK–MB, and CK–BB. CK is abundant in tissues with elevated energy transfer such as skeletal muscle, myocardium, and brain. In other visceral tissues [28], noticeable CK concentrations can be found in the uterine tissue and in every inner organ [25]. The serum of healthy cows contains almost entirely CK-MM, while inner organs contain mostly CK-BB. Mechanical and metabolic stress of the uterine tissue is known to cause elevated CK activities before and after normal parturition in cows [29]. Furthermore, serum concentrations of CK 3 days after parturition are lower in healthy Holstein cows (median of 121 U/l) than in cows with retained placenta (median 175 U/l), dystocia (median 310 U/l), milk fever (median of 385 U/l) [2], and abomasal displacement. [25]. However, elevated CK serum concentrations can be expected whenever recumbency occurs, due to the neuromuscular damage [30]. Weber et al. (2019) pointed out that recumbent Holstein cows show higher CK serum concentrations than healthy ones at day 5 after parturition (mean of 5011.28 ± 13386.53 vs 666.44 ± 1645.44) [31]. As for endometritis, CK has been assessed in dairy cows [25] and in Iraqi buffalo cows [24]; results showed that animals with endometritis had higher CK activity than healthy ones. However, higher CK blood concentration were found in estrous beef cows than in non-estrous ones [32].

To the best of our knowledge, CK has never been investigated as a diagnostic tool for endometritis in beef cows. The first objective of this study is to define the basal CK serum concentrations of healthy Piedmontese beef cows, the second consists in the evaluation of the accuracy of CK serum concentrations in detecting clinical endometritis.

2. MATERIAL AND METHODS

2.1 Animals enrollment

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

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

Two-hundred and three (203/264) cows belonging to the latter group were deemed as healthy (HEALTHY), 33 cows (33/264) required a number of artificial insemination (AI) higher than 3, without presenting any uterine pathologies and were defined as repeat breeding cows (RB), whereas another group included 28 (28/264) cows diagnosed with clinical endometritis (ENDO). These cows were examined at 30±5 days post-partum and sorted into the HEALTHY or ENDO group according to the result of the physical examination, which was always performed by the same veterinarian.
Vaginal discharge was categorized as described by Williams et al. (2005), using a 4-point classification system: 0 = no or clear mucus, 1 = mucus containing few flecks, 2 = discharge containing less than 50% pus, 3 = discharge containing more than 50% pus. A blood sample was collected from each animal during the clinical examination. All cows were submitted to AI based on heat detection at 60±5 days postpartum.

2.2 Blood samples collection and biochemical analysis

Blood samples were collected by venipuncture from the coccygeal vein using an 8 ml evacuated serum collection tube and a 20 G needle (Vacutainer® Venoject, Terumo, Leueven, Belgium); the samples were immediately refrigerated and transported to the laboratory within 4 hours. The blood was centrifuged at 2,000 rpm for 10 minutes and the serum was separate and stored at -20°C in 1 ml SafeLock tubes (Eppendorf®, Hamburg, Germany).
CK was measured with a clinical chemistry analyzer KUADRO® BPC (Biosed s.r.l, Rimini, Italy) with Creatine Kinase immunologic kinetic UV-test (MTD Diagnostics, Caserta, Italy) in accord with International Federation of Clinical Chemistry (IFCC).

2.3 Statistical analysis

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

Afterwards, HEALTHY (including RB) and ENDO cows were analyzed with a one-way ANOVA statistical method between healthyRB (HEALTHY + RB) and diseased (ENDO) animals and also by each status (HEALTHY, RB, ENDO) to point-out any difference in CK serum concentrations. Furthermore, a one-way ANOVA statistical method was used to evaluate reproductive performances such as partum-to-conception interval (PC) and number of AI among groups. Bonferroni pot-hoc test was used for pairwise comparison.

A receiver operating characteristic (ROC) curve model (pROC) and the area under the curve (cvAUC) were calculated to find the optimal CK cut-off point for evaluating clinical endometritis at 30 days pp and infertility (PC at 120 and 150 days and number of AI).

Data were indicated as mean ± ds. P values ≤ 0.05 were considered significant, and trends were considered to be present at P values between 0.06 and 0.08. Statistical analyses were performed using R statistical software (ver. 2.15.2).

3. RESULTS

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

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

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

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

4. DISCUSSION

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

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

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

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

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

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

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

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

5. CONCLUSION

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

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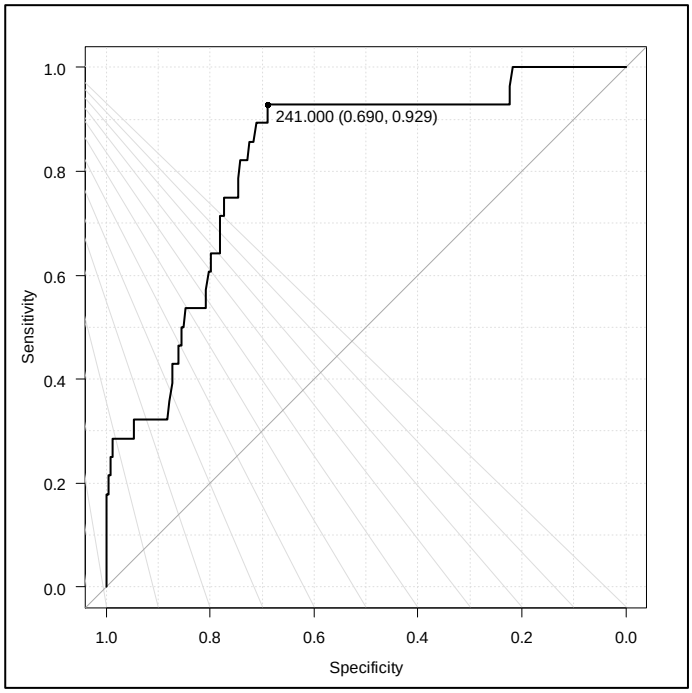

Fig 1. ROC curve indicates a cut-off of 241 U/L for CK to predict endometritis. Sensitivity 92%, Specificity 69% and AUC 0.81.

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.

354