Comparison of blood metabolites and GSH-Px, SOD, MDA levels as a predictor of pregnancy in primiparous cows after the Presynch-Ovsynch protocol

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SUMMARY

The reactive oxygen species level and antioxidants have determinative roles in gamete development, steroid synthesis and fertilization. The objective of the current study was to compare some metabolic and antioxidant parameters in primiparous cows with and without pregnancy after the presynch-ovsynch protocol in the postpartum period. Sixty dairy cows were allocated to two groups according to their pregnancy status after timed artificial insemination following the presynch-ovsynch protocol. Blood samples were collected at the day of presynch-ovsynch protocol started, at AI and at pregnancy examination to determine glucose, urea, total protein, phosphorus and calcium, GSH-Px, SOD and MDA levels. Receiver operating characteristics (ROC) curves were used to determine the cow-level thresholds for the subsequently pregnant. Moreover, pairwise comparisons were made of the area under the curve (AUC) of ROC curves for the thresholds of GSH-Px, SOD and MDA for identifying the cows most likely to conceive. The biochemical metabolites were in physiological ranges. Serum glucose concentration was greater at the beginning of presynch-ovcsynch in cows that became subsequently pregnant. An interaction was noted between group x time for serum SOD, MDA and GSH-Px levels. The MDA concentration was lower before the synchronization protocol in cows that became subsequently pregnant. Although GSH-Px concentration was greater, SOD was lower after the synchronization sampling time in cows that became subsequently pregnant. MDA and SOD concentrations changed over time in cows that became subsequently pregnant. MDA level at the beginning of synchronization was the best predictor for identifying the cows most likely to conceive with AUC values of 0.866. In conclusion, while presynchronization MDA and post synchronization SOD were lower, post synchronization GSH-Px were higher in pregnant cows. MDA level at the beginning of presynch-ovsynch was the only risk factor for pregnancy. Thus, MDA levels before the synchronization might be used as a biomarker for selecting the cows for presynch-ovsynch protocol.

KEY WORDS

Antioxidant; oxidant; pregnancy; presynch; timed artificial insemination.

INTRODUCTION

The key component of the dairy farming industry is profitability; which optimal reproductive performance directly depends. For the genetic gains of milk yield; aggressive efforts made in dairy herds led to a decrease in estrus detection rates impaired fertility[1]. Not only the milk yield but also diseases and management such as diet, energy status, liver function associated with fertility parameters directly influence uterine and oocyte environment as well as embryo development[2].

In the postpartum period, a healthy uterine environment is one of the major requirements for a successful pregnancy. However, the efficiency of reproduction in lactating cows is not optimal; this situation results in the development of different reproductive management strategies focused on improving pregnancy rates. One of these strategies which is a combination of presynchronization and ovsynch (Presynch-Ovsynch) could make enable to manage the time of the first postpartum insemination[3].

Cows suffer from excessive production of free radicals. Any imbalance between the production and neutralization of free radicals results in oxidative stress[4]. Reactive oxygen species (ROS) and antioxidant scavenging systems are detected in the female genital tract[5]. The level of ROS and antioxidants have a regulatory role in oocyte maturation, steroidogenesis and fertilization[6]. On the other hand, antioxidants are necessary for the prevention of reproductive disorders[7]. The adverse effect of oxidative stress on the reproductive tract includes the damage of

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the DNA of oocyte, ovary and endometrium[6].

There are different roles for antioxidant enzymes. As a consequence of oxidative stress, the final production of lipid peroxidation called malondialdehyde (MDA) increases[8]. In contrast, superoxide dismutase (SOD) converts superoxide anion to hydrogen peroxide and oxygen and minimizes the production of hydroxyl radicals[9]. On the other hand, glutathione peroxidase (GSH-Px), the other antioxidant enzyme, is the enzyme involved in the degradation of hydrogen peroxide formed by the SOD reaction[10]. At the molecular level, the concentration of these enzymes in follicular fluid and their effects on human fertility is known[11]. The use of blood concentrations of these antioxidant enzymes as a marker for selecting suitable animals for the herd level synchronization protocol requires further research.

Antioxidants are closely related to fertility. The effectiveness of oxidants, which play a role in oocyte development and the formation of fertile embryos of follicles that become dominant in a short time as a result of hormonal programs in cows is unknown. It was hypothesized that blood concentrations of metabolites, MDA, SOD and GSH-Px enzymes might be used as screening biomarkers for selecting cows for synchronization protocol in herd level. The present study aimed to compare some metabolic and antioxidant parameters in primiparous cows with and without pregnancy after the presynch-ovsynch protocol in the postpartum period also evaluated antioxidant parameters usability as screening biomarkers before and after synchronization protocol.

MATERIALS AND METHODS

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Table 1 - Integrant of diets during the study period.

Component	Ingredient. % of DM
Vetch hay	-
Corn flake	7.09
Alfalfa hay	12.89
Corn silage	54.12
Wheat straw	2.06
Soybean pulp 8% Crude Protein	3.22
Concentrated feed	20.62
Limestone	1.57
Salt	0.26
Ammonium chloride	-
Dicalcium phosphate	0.44
Magnesium oxide	0.44
Magnesium sulphate	0.25
Sodium bicarbonate	0.7
Calcium sulphate	0.1
Mineral-vitamin mix[1]	0.2

[1]Contained a minimum of 4.3%Mg, 8% S, 6.1% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5000 mg/kg Cu, 250 mg/kg of I, 40 mg/kg of Co, 150 mg/kg Se, 2200 kIU/kg of vitamin A, 660kIU/ kg of vitamin D_3, and 7700 IU/kg of vitamin E.

Animals, housing and management

This was a retrospective cohort study conducted on a commercial dairy farm in Ankara, Turkey. A total of 60 primiparous Holstein dairy cows were enrolled in the study. Cows were housed together, milked twice daily and milk yield was measured during the study period. The barns were naturally ventilated and had artificial lighting. Cows were fed with TMR twice daily for ad libitum intake and free access to water. TMR was formulated by a professional nutritionist daily and balanced to meet dietary requirements for a lactating dairy cow according to body weight and milk production (Table 1).

Study design

Animals were enrolled in study 30 (±4) days after parturition. Animals were divided into two groups according to pregnancy status at 134 DIM as pregnant (n=29) and non-pregnant cows (n=31). Healthy cows according to postpartum reproductive examination were subjected to the presynch-ovsynch protocol 40 days after parturition (Figure 1). All cows were monitored for estrus signs by a trained person twice daily (Morning-evening) at least for 45 minutes after the PGF2α injection of ovsynch protocol. The estrus response was calculated as previously described. Behavioral signs and uterine tone were used for the determination of estrus intensity scores which were as follows: 1 = poor, no uterine tone with no behavioral signs; 2 = satisfactory, mild uterine tone, slight mucus discharge, some restlessness; 3 = good, intermediate uterine tone, mucus discharge, restlessness, nervousness; 4 = very good, good tone, stand to be mounted, vulvar swelling, thick mucus discharge, restlessness; 5 = excellent, high tone, stand to be mounted, thick mucus discharge, restless[12]. For the first service, all cows were inseminated with commercial frozen bull semen by the rectovaginal method. Inseminations were conducted 16 h after the last GnRH injection by timed artificial insemination (TAI). The same person performed two transrectal USG examinations at 104 days and 132 days postpartum for the diagnosis of pregnancy.

Blood collection and analysis

Bloods (10 ml) were collected before morning feeding on the coccygeal vein or artery into vacutainer tubes that contain no preservatives (Vacutainer, Betcon Dickinson, USA) at the day of presynch-ovsynch protocol started, the day of TAI, and the day of pregnancy check (Figure 1). After the collection of samples, tubes were placed on ice and centrifuged (3000×*g* 15 °C, 15 min) within one hour. Then serum samples were transferred to a new tube for storage and stored at −20 °C until analysis.

Serum samples were analyzed for the concentrations of glucose, urea, total protein, phosphorus and calcium by using auto-analyzer (Erba XL 600). Serum SOD activity was assessed using the method of Sun et al.[13]. Moreover, MDA levels were analysed with the method described by Ohkawa et al.[14]. Furthermore, GSH-Px activity was determined by the method of Sedlak and Lindsay[15]. The results of SOD, MDA and GSH-Px were expressed as IU/L, μmol/L and μmol/L, respectively.

Environmental data

Temperature and relative humidity inside the barns were recorded daily. Daily maximum and minimum temperatures and humidity index (THI) were calculated according to Aguilar et al.[16]. THI < 72 was indicative of no heat stress.

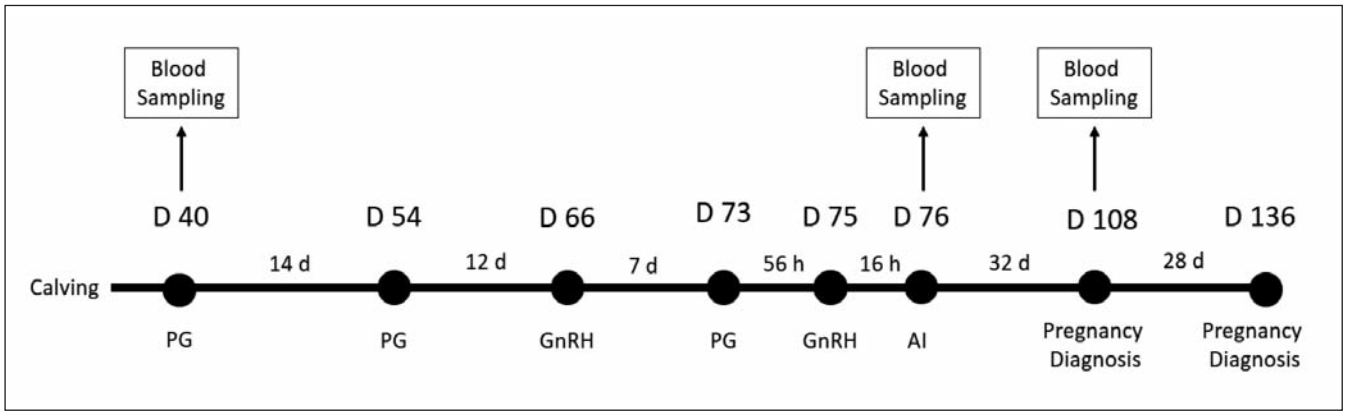

Figure 1 - Briefly, day 40, cows received PGF2α (500mcg, Enza-prost, Ceva, Turkey) and blood sampling were performed. Fourteen days later second PGF2α injected. Twelve days after the last PGF2α, ovsynch protocol started. On the day of insemination and pregnancy diagnosis blood samples were collected for determination of some selected serum metabolites and SOD, GSH-Px, MDA levels.

Statistical analysis

This was an observational cohort experiment. Cows were enrolled in weekly cohorts of 5 to 7 animals. Estrus intensity was compared by using Mann-Whitney U test. The correlation between metabolites and ions was evaluated. Descriptive statistics for each variable were calculated and presented as "Mean ± Standard Error of Mean". All data were analyzed using MIXED procedure of SPSS (V23.0; SPSS Inc., Chicago, IL, USA). The effect of status of pregnancy, time of sampling and their interaction on SOD, MDA, GSH, glucose, urea, total protein, phosphorus and calcium were analyzed by using the following model with repeated measures:

$$Y_{ijk} = \mu + G_i + D_j + (G \times D)_{ij} + e_{ijk}$$

Where, Y_{ijk}, dependent variable; μ, overall mean; G_i, the effect of the status of pregnancy (i = Pregnant and nonpregnant); D_j, the effect of time of sampling (j = Before synchronisation and After synchronisation); $(G \times D)_{ij}$, the interaction between group and day of sampling; and e_{ijk}, residual error.

Animals within the group were assessed as a random effect, while the status of pregnancy, time of sampling and their interaction were assessed as a fixed effect. P<0,05 was considered significant in all analyses. When a significant difference was revealed, any significant terms were compared by Simple effect analysis with Bonferroni adjustment.

Receiver operating characteristic (ROC) curves were obtained using MedCalc version 9.2.0.1. The concentration of SOD, MDA and GSH were evaluated by ROC analysis to determine a critical threshold for predicting and identifying the cows most likely to conceive at each sampling time. The higher area under the curve (AUC) values related to each metabolite were determined as the most predictive critical threshold for the identification of cows most likely to conceive. Besides, sensitivity, specificity and likelihood ratios (LRs) were calculated.

RESULTS

Estrus response in cows did not differ ($P>0.05$) and was 86.2% (25/29) in cows that became subsequently pregnant and 80.6% (25/31) in cows that became subsequently non-pregnant. Similarly, the means of estrus intensity remained insignificant ($P>0.05$) and was 2.78±0.11 in cows that became subsequently pregnant, and 2.71±0.13 in cows that did not subsequently become pregnant. The percentage of endometritis in each group were similar (4/29 for subsequently pregnant and 5/31 for subsequently non-pregnant cows respectively). In the present study, the pregnancy status of all cows was re-checked twice (Figure 1). Moreover, there was no embryonic loss in any of the cows during the study period.

The average minimum and maximum THIs during the study period were 60.91(±8.23) and 62.71 (±7.44) respectively. During the study period, none of the cows experienced a Max THI greater than 72. Although the average milk yield for 40-day was similar (29±8.81- 28±8.73 liter per day for pregnant and non-pregnant cows, respectively), there was no significant difference between the two groups. Furthermore, body condition scores were similar for pregnant and non-pregnant cows.

Serum metabolites

The level of serum glucose concentration was greater at the beginning of the breeding program in cows that became subsequently pregnant. No cows had hypoglycemic status during the measure points. After the synchronization program, serum glucose concentrations were similar among groups. Besides, there was no interaction noted for serum calcium, phosphorus, urea and total protein concentration in both groups (Table 2). On the other hand, an interaction was noted between group x time for serum SOD, MDA and GSH-Px levels. The MDA concentration was lower at the beginning of the synchronization protocol in cows that became subsequently pregnant. Although GSH-Px concentration was greater, SOD was lower after the synchronization sampling time in cows that became subsequently pregnant. MDA, SOD and GSH-Px concentrations changed over time in cows that became subsequently pregnant (Table 3).

Clinical thresholds

ROC analysis was performed to determine critical thresholds (i.e., highest sensitivity and specificity) for SOD, MDA, GSH-Px in order to predict the subsequent pregnancy. The results of ROC curve analysis for the determination of clinical thresholds, specificity, sensitivity, AUC, LR and P values are presented in Table 4. The LRs were determined based on critical thresholds calculated by univariable ROC analysis. For instance, LR positive (LR+) reflects the likelihood that a positive test re-

Table 2 - Mean serum glucose, phosphorus, calcium, urea, total protein concentrations between subsequently pregnant and non-pregnant cows during the measurement points (mean±SEM).

Time of Sampling Parameters	Before the Synchronization	At AI	After Synchronization	*p*-value Group	 Time	 G*T
Glucose (mg/dL)						
Pregnant cows	61.76±1.89$^{a, A}$	58.15±1.25^{ab}	57.63±1.18^{b}	0.092	0.000	0.036
Non-pregnant cows	53.76±1.92$^{b, B}$	54.95±2.21^{ab}	56.89±2.25^{a}			
Phosphorus (mg/dL)						
Pregnant cows	5.64±0.48^{b}	6.14±0.16^{ab}	7.35±0.28^{a}	0.288	0.021	0.242
Non-pregnant cows	6.02±0.64	5.98±0.41	6.22±0.34			
Calcium (mg/dL)						
Pregnant cows	8.78±0.29	8.81± 0.17	8.69±0.11	0.472	0.621	0.328
Non-pregnant cows	8.91± 0.19	8.71±0.21	8.79±0.14			
Urea (mg/dL)						
Pregnant cows	31.93±1.91	32.1±1.85	31.70±1.23	0.555	0.412	0.354
Non-pregnant cows	31.16±1.82	31.74±1.89	31.82±1.62			
Total protein (g/dL)						
Pregnant cows	7.23±0.83^{c}	7.18±0.43	7.21±0.26^{a}	0.616	0.753	0.344
Non-pregnant cows	7.16±0.66^{c}	7.21±0.39	7.38±0.21^{a}			

a, b, c: Differences between averages in the same line with different letters are statistically significant (p<0,05).
A, B: Differences between averages in the same column with different letters are statistically significant (p<0,05).

sult (i.e., value equal or under the threshold) could come from an animal that became subsequently pregnant in comparison to non-pregnant. Moreover, ROC curves for the critical threshold for MDA, GSH-Px and SOD predicting the pregnancy are shown in Figure 2. For predicting the pregnancy at the beginning of synchronization, the ROC curve for MDA presented a higher AUC of 0.866, when compared with those of SOD (0.612), GSH (0.545). On the other hand, after the synchronization, the ROC curve for SOD and GSH presented a higher AUC of 0.964 when compared with MDA (0.527). The MDA concentration at the beginning of synchronization and GSH and SOD at the end of synchronization were the best predictors for identifying cows most likely to conceive.

DISCUSSION

The present study compared some metabolic and antioxidant parameters in primiparous cows with and without pregnancy after the presynch-ovsynch protocol in the postpartum period. Moreover, this study evaluated antioxidant parameters usability as screening biomarkers both before and after the synchronization protocol.

The estrus response was similar to data previously published by Hassan et al.[12]. The time of estrus was similar among groups The possible reason for the estrus response being lower than 100 % might be related to the follicular wave difference due to the second GnRH injection. It means the absence of ovulatory follicles[12]. Ovulation rates were not determined, but this is a fact that 29 of all cows ovulated successfully. On the other hand, all cows were examined before the study period and they had at least one luteal tissue in their ovaries before including to the study.

The milk yield and metabolism of dairy cows are one of the important factors. High yielding cows are less likely to become pregnant in comparison to low yielding cows. Although there is an antagonist relationship between milk production and fertility, there are also studies that state otherwise[17]. However, the milk yield was not an effective factor in the present study, because it was similar among groups during the study period. There are some preconditions for maintaining good reproductive performance, such as the health of the cow and nutritional management[18]. It is a fact that postpartum antioxidant

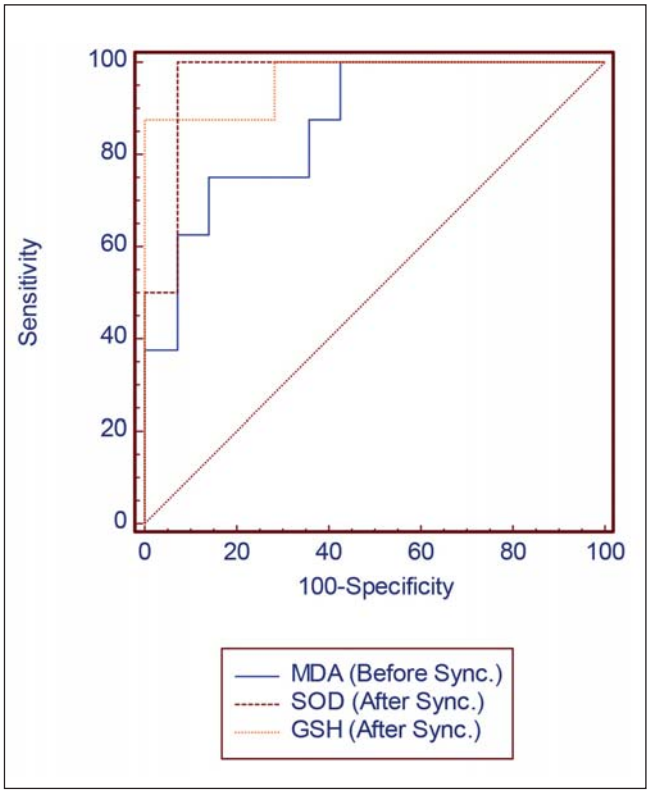

Figure 2 - Display of ROC curves that determined clinical threshold for concentrations of MDA before synchronization (≥51.09 mmol/L; doded line). SOD (≥1.69 IU/L, dashed line) and GSH (≥784 mmol/L, solid line) after synchronization predicting cows most likely to conceive. The diagonal line represents the specificity and sensitivity level at which the test informative (ROC, receiver operating characteristic; AUC, area under the curve; MDA, malondialdehyde; SOD, superoxide dismutases; GSH, glutathione peroxidase; SE, standard error; CI, confidence interval).

Table 3 - Mean Superoxide dismutase (SOD), Malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) levels between subsequently pregnant and non-pregnant cows during the measurement points (mean± SEM).

Parameter	Status of pregnancy	Before Synchronization	Time of sampling At AI	After Synchronization	*p*-value Status	Time	S*T
SOD (IU/L)	Pregnant	1.79 ± 0.03 ^{a}	1.67±0.04 ^{ab}	1.53 ± 0.05 $^{b, B}$	<0.001	0.003	<0.001
	Non-pregnant	1.81 ± 0.02	1.84±0.02	1.87 ± 0.03 ^{A}			
MDA (µmol/L)	Pregnant	45.77 ± 7.03 $^{a, B}$	40.55±6.54 ^{ab}	31.82 ± 4.73 ^{b}	0.008	0.001	0.010
	Non-pregnant	133.29 ± 23.03 $^{a, A}$	57.11±5.71^{ab}	33.48 ± 4.24 ^{b}			
GSH (µmol/L)	Pregnant	592.07 ± 71.37 ^{b}	722.42±58.15 ^{ab}	806.14 ± 21.30 $^{a, A}$	0.132	0.781	0.005
	Non-pregnant	686.62 ± 72.62 ^{a}	604.14±66.12 ^{ab}	509.39 ± 52.09 $^{b, B}$			

a, b, c: Differences between averages in the same line with different letters are statistically significant (p<0,05).
A, B: Differences between averages in the same column with different letters are statistically significant (p<0,05).

status and metabolite levels are the factors that might help to evaluate the success of postpartum health and the quality of nutrition. Therefore, for the elimination the effect of the nutrition, all cows were fed with the same TMR, which were prepared according to the requirements during the postpartum period.

The key to successful reproductive management depends on many factors: However, at the cell level, it depends on oocyte and sperm quality. The macro and microenvironment of follicle oocytes are influenced by the metabolism of the cow. Alterations on the level of metabolites in blood serum reflects to the follicular fluid of the dominant follicle[19]. The low glucose concentration, which has a toxic effect on oocyte during the maturation process, blocks cumulus expansion and decreases developmental competence. Glucose is metabolized via glycolytic pathway to the pyruvate and lactate which are key factors to produce ATP used by oocyte. In vitro studies showed that glucose is a very important metabolite for the maturation and cleavage and blastocyst formation of the embryo. Decreased concentration of glucose impairs those stages and reduces the quality of oocyte[20]. On the other hand, oocyte and follicular development process starting from primordial follicle to the mature ovulatory stage takes 80 to 100 days in cows. However, by using hormonal treatment, the duration of this process could be shortened and taken into a limited time. In our opinion, metabolic status is more effective for the development and quality of follicle and oocyte during this limited period. The present study showed that cows that became pregnant after the synchronization protocol had greater glucose concentration. Although non-pregnant cows showed lower glucose concentration in comparison to pregnant cows, the glucose level was not in the hypoglycemic state. At the beginning of reproductive management, the comparison of serum glucose concentration might be helpful for selecting the cows to the reproductive management program. Furthermore, the fact that protein, urea, and calcium-phosphorus levels were similar for both groups showed that the cows included in the study were metabolically healthy.

The decreased activity of SOD in cows that subsequently became pregnant might be under the influence of the complex relationship between body and embryo. It has been documented that SOD and other antioxidant activities changed due to increased fetal requirements at later stages of pregnancy for the protection of placenta and fetus against increased superoxide anion and hydrogen peroxide levels[21]. Similar to that, the possible explanation for decreased SOD levels might be the usage of this enzyme by the body for the protection of embryonic development. Ovarian steroids might modulate the SOD activity in cows as shown in rats[22]. In our opinion, there is a complex relationship between the oxidant-antioxidant enzyme mechanism and hormone levels. Thus, decreased SOD activity might be related to hormonal changes. Because during that time progesterone levels increased in pregnant cows. Besides, progesterone enhances ROS production[23]. The increase in ROS under influence of progesterone might increase the usage of SOD, thus, the level of SOD might decreased in the blood. Considering all this information, the mechanism can be determined exactly by monitoring progesterone levels and antioxidant enzyme activity during the synchronization process.

It is not possible to mention the same mechanism for the GSH-Px. Progesterone does not alter the level of GSH-Px[23]. The present study showed that GSH-Px level increased in pregnant cows

Table 4 - Receiver operating characteristic (ROC) curve analysis for the determination of critical thresholds for SOD, MDA, GSH-Px as predictors identification of cows most likely to conceive. The results of ROC curve analysis of critical SOD, MDA, GSH-Px thresholds for prediction of cows most likely to conceive tabulated and ranked by their respective area under curve (AUC). Data about the levels on SOD, MDA, GSH-Px showing sensitivity, specificity, and LR+,- are also provided.

Metabolite	Days	Threshold	Se	%95 CI for Se	Sp	%95 CI for Sp	LR (+ or -)	AUC	p
SOD (IU/L)	Before Synchronization	1.73	50.0	16.0 - 84.0	92.86	66.1 - 98.8	(+) 7	0.612	0.370
MDA (µmol/L)		51.09	75.0	35.0 - 96.1	85.71	57.2 - 97.8	(+) 5.25	0.866	<0.001
GSH (µmol/L)		799	100.0	62.9 - 100.0	21.43	4.9 - 50.8	(+) 1.27	0.545	0.730
SOD (IU/L)	After Synchronization	1.69	100.0	62.9 - 100.0	92.86	66.1 - 98.8	(+) 14	0.964	<0.001
MDA (µmol/L)		42.39	87.5	47.4 - 97.9	42.86	17.8 - 71.1	(+) 1.53	0.527	0.837
GSH (µmol/L)		784	87.5	47.4 - 97.9	100.0	76.7 - 100.0	(-) 0.13	0.964	<0.001

during the study period and was greater than non-pregnant cows. GSH-Px is an important enzyme for successful embryonic development[24]. It is already known that cessation of GSH-Px biosynthesis resulted in embryonic death in mice[25]. Based on this, the increased level of GSH-Px might be a useful biomarker for evaluating the embryonic status of dairy cows. ROC analysis also supports this hypothesis, pregnant cows showed greater AUC level for GSH-Px level after the synchronization in compare to other markers. However, more comprehensive studies should be conducted to evaluate the in vivo embryo viability and maternal concentration of GSH-Px relationship. The last product of lipid peroxidation, MDA, could be a useful biomarker for the determination of oxidative stress[26]. Different studies have been conducted for the determination of the role of MDA levels on cow health, however, these studies examined late lactation and a transition period[27,28]. To our knowledge, this is the first study that determined the role of antioxidant enzymes on pregnancy before and after the synchronization. In the present study, cows that became subsequently pregnant after the presynch-ovsynch method showed lower MDA level at the beginning of synchronization. The level of MDA was similar at insemination and after the synchronization among groups. This situation means MDA decrease might not be related to pregnancy status; it may depend only on progesterone level. Furthermore, a greater level of MDA at the beginning of synchronization might negatively affect the follicle development as well as the quality of oocytes in cows. However, further research is needed to evaluate the effect of MDA on follicle development and oocyte quality in cows. On the other hand, the increased concentration of MDA might be the reflection of the increased lipid peroxidation only for a short period of time[27]. MDA levels negatively correlated with fertilization rate and had a negative impact on embryo quality in human IVF studies[29]. In previous studies on oocyte level, it has been reported that the level of MDA is a useful marker for the prediction of the outcomes of assisted reproductive techniques[30]. Similarly, the present study showed that MDA concentration is a useful biomarker for the selection of cows including the synchronization protocols.

CONCLUSIONS

In conclusion, cows that became subsequently pregnant showed greater serum glucose concentration at the beginning of synchronization. Pregnant cows showed lower MDA levels before the synchronization while the SOD level was lower after the synchronization. Moreover, GSH-Px was greater in pregnant cows after the synchronization. MDA level at the beginning of presynch-ovsynch was the only risk factor for pregnancy. Thus, higher MDA levels before the synchronization might affect the quality of oocytes negatively and can be used as a biomarker for selecting the cows for presynch-ovsynch protocol.

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