Pregnancy Rates Associated with Oxidative Stress after Estrus Synchronization of Bulgarian Murrah Buffaloes in Breeding and Non-Breeding Season

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Abstract: *Background*: The current study aims to measure the effect of oxidative stress on the pregnancy rates of Bulgarian Murrah buffaloes during the breeding and non-breeding season.

Methods: The study group consisted of 24 mature buffaloes more than 40 days after parturition. The following parameters were measured: Reactive Oxygen Species (ROS) products, Ascorbate radicals, Malondialdehyde (MDA), Nitric Oxide (NO), Super Oxide Dismutase (SOD), Glutathione peroxidase (GSH-Px), Protein Carbonyl Content (PPC), and total Nitric oxide. The Presynch/Ovsynch protocol was used for estrus synchronization.

Results: A statistically significant increase in ROS products were measured in blood serum during the breeding season compared with the non-breeding season. The highest levels measured were in non-pregnant buffaloes during the breeding season. High levels of oxidative stress were registered due to low SOD activity in buffaloes during the breeding season compared to SOD activity during the non-breeding season. The highest SOD activity was observed in non-pregnant buffaloes during the summer season. The lowest GSH-Px levels were observed in non-pregnant buffaloes during both study periods. During the breeding season, concentrations of total NO and PPC were elevated.

Conclusion: Comparing the obtained results for oxidative stress and antioxidant activity concerning pregnancy rate depending on the season showed that pregnancy in buffaloes during the breeding season was realized at higher values of NO and SOD. Increased oxidative stress was observed, resulting in a statistically significant increase in serum ROS products, as well as decreased SOD activity in buffaloes during the breeding season.

Keywords: Oxidative stress, biomarkers, pregnancy rate, Buffaloes, season.

INTRODUCTION

Reactive oxygen species (ROS) are products of normal oxygen metabolism and play an important role in a number of physiological processes, such as phagocytosis, cell differentiation, and others. ROS are formed continuously as normal by-products of cellular metabolism and in low concentrations are essential for many physiological processes, such as protein phosphorylation, activation of transcription factors, cell differentiation, apoptosis, egg maturation, steroidogenesis, cellular immunity, and cell defense against microorganisms. When the ROS concentrations increase above the physiological level, oxidative stress is reached, causing adverse effects [1]. Moreover, oxidative stress has a negative effect on the reproductive process in mammals [2]. In addition, mitochondrial damage and apoptotic mechanisms triggered by ROS damage the oocytes and endometrium, thereby contributing to reduced fertility in dairy cows [3]. Therefore, the assessment of redox homeostasis in the blood increasingly contributes to the knowledge of the processes involved in reproductive and metabolic disorders as a complementary tool for assessing the health and metabolic status of animals [4,5]. To prevent pathological changes, it is desirable to determine the ROS level. Oxidative stress can be particularly dangerous due to the absence of clear clinical symptoms. The effect of oxidative stress in cyclic and repeat-breeding cows was established by Ali *et al*. [6]. Seasonal variation (summer and winter) of oxidative stress and its effect on fertilization in Murra buffaloes was reported by Kumar *et al*. [7]. The authors found that MDA concentrations increased in summer compared to the winter season and concluded its effect on pregnancy rates in buffaloes. Recorded GSH-Px levels were similar in the summer and winter seasons in pregnant and non-pregnant buffaloes. During the summer season, due to thermal stress, reduced SOD values were found compared to winter in Egyptian

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buffaloes [8]. Given the above information in the present study, the aim is to establish the oxidative status in relation to pregnancy rates in buffaloes of the Bulgarian Murrah breed after estrus synchronization during breeding and non-breeding season.

MATERIAL AND METHODS

Animals, Breeding, and Feeding

The animals were kept on the buffalo farm of the Agricultural Institute, Shumen (Northeastern Bulgaria, latitude: 43.28N, longitude: 26.93E). The ration included concentrated fodder for lactating buffaloes, beer porridge, alfalfa hay, straw, and constant access to water. The study included 24 multiple buffaloes born over 40 days after birth from the Bulgarian Murrah breed. The buffaloes weighed from 550 kg to 700 kg, aged from 4 to 8 years, and lactation milk 1800 - 2100 kg with daily double milking. The experimental animals included in the study were with BCC 4-5. They had normal calving and no gynecological problems during the postpartum period. The treatment was performed in two groups – group B1 during the breeding season (autumn-winter n = 16) and group B2 in the non-breeding season (spring-summer n = 8).

Ethical Approval

The experiment was approved by the Animal Ethics Committee to the Faculty of Veterinary Medicine, Trakia University - Stara Zagora, in compliance with the minimum requirements for the protection and welfare of experimental animals according to Ordinance No. 20/1.11.2012 of the Ministry of Agriculture and, Food and Forestry Food from November 1, 2012, Republic of Bulgaria.

Hormone Treatment and Blood Tests

Blood samples to determine the indicators of oxidative stress were obtained from *v. jugularis* by means of a vacuum blood collection system on the day of starting the estrus synchronization program. Subsequently centrifuged, the serum was stored at -20°C until the study.

Presynch/Ovsynch protocol was applied: day 0 500 UI Synchrostim (Equine serum Gonadotropin, Ceva Sante Animale, France) + 25 mg Enzaprost (Dinoprost trometamol, Ceva Sante Animale, France), 3 and 10 days 100 μg Ovarelin (Gonadorelin, Ceva Sante, France), 17 days 25 mg Enzaprost (Dinoprost trometamol, Ceva Sante Animale, France), 19 days 100 μg Ovarelin (Gonadorelin, Ceva Sante Animale, France) and artificial insemination with a fixed time 16 hours after the last treatment with GnRH.

Immuno-Enzymatic Methods

Determination of the activity of the antioxidant enzyme superoxide dismutase (SOD), Reduced glutathione (GSH-Px), Total nitric oxide (NO), and protein carbonyl content (PCC). Standard ELISA kits were used to analyze these biomarkers. All enzyme-linked immunosorbent assays were performed according to the procedure described in the respective kit.

Spectrophotometric

Determination of Lipid Peroxidation Products Measured as Malondialdehyde (MDA)

The thiobarbituric acid (TBA) method measures the malondialdehyde (MDA) reactive products used. Briefly, 1 ml of plasma, 1 ml of saline, and 1 ml of 25% trichloroacetic acid were mixed and centrifuged at 7000 rpm for 20 minutes. Two milliliters of protein-free supernatant were mixed with 0.5 ml of 1% TBA and heated at 95 ° C for 1 hour. After cooling, the pink color intensity of the final product fraction at 532 nm was recorded spectrophotometrically. The MDA concentration is calculated by the following formula:

$$1\mu mol/1MDA = \frac{OD532 \times 1.75}{0.156}$$

where OD532 is the optical density at 532 nm and extinction = 1.56×10^5 M^{-1} cm^{-1}.

EPR Methods

EPR measurements of all tested samples were conducted at room temperature (18-23°C) on an X-band EMX^{micro}, spectrometer Bruker, Germany, equipped with a standard Resonator. Quartz capillaries were used as sample tubes. The sample tube was sealed and placed in a standard EPR quartz tube (*i.d.* 3 mm) which was fixed in the EPR cavity. All EPR experiments were carried out in triplicate and repeated. Spectral processing was performed using Bruker WIN-EPR and SimFonia software.

Ex Vivo EPR Method for Testing the Level of Ascorbate Radicals in Serum

For the study of Ascorbate (Asc) radicals, we used the method of Bailey [9] with modifications made by us.

Ex Vivo EPR Evaluation of the Levels of Serum ROS Products

To demonstrate real-time lipid peroxidation, serum ROS levels were determined by the ex vivo EPR method of Shi *et al*. [10] with modifications by Zheleva *et al*. [11].

Ex Vivo Determination of the NO Radicals Level in Serum

Based on methods published by Yoshioka *et al*. [12] and Yokoyama *et al*. [13], we developed and adapted an EPR method to determine nitrogen levels.

Ultrasound Pregnancy Diagnosis

The ultrasound examination was performed with a SonoScape S2 Vet device (SonoScape Co. LTD, Shenzhen, China), a multifrequency (7-12 MHz) linear probe, and a transrectal approach on day 35 after fixed insemination with a fixed time for early pregnancy diagnosis.

Statistical Analysis

The results were processed on a statistical computer program Statistica 7, using a non-parametric method to compare averages. The results are presented as Mean, Standard Deviation (SD), and confidence levels. Differences were considered statistically significant at $p<0.05$.

RESULTS

The results of the studied indicators of oxidative stress and antioxidant activity of buffaloes in different seasons are presented in Table **1**.

A statistically significant increase in ROS products in blood serum was found in buffaloes during the breeding season, compared to the values of ROS products in the non-breeding season. Concentrations of ascorbate radicals were twice as high in buffaloes during the summer season and especially in non-pregnant buffaloes.

Increased oxidative stress due to decreased SOD activity in buffaloes during the breeding season was recorded, compared to the results for SOD activity during the non-breeding season. The highest activity is reported in non-pregnant buffaloes during the summer season.

Reduced glutathione (GSH-Px) concentrations are high in buffaloes with established pregnancies in both seasons. The lowest levels were recorded in non-pregnant animals during both study periods.

Comparing the obtained results for oxidative stress and antioxidant activity with respect to pregnancy rate depending on the season, it was found that pregnancy in buffaloes during the breeding season was realized at higher values of NO and SOD.

DISCUSSION

The level of ROS products is considered an indicator of free radical production. ROS products include oxygen-containing not only free radicals such as superoxide anion and hydroxyl radical but also some non-radical oxygen derivatives, e.g., hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) [14]. Increased metabolic activity leads to increased

Table 1: Levels of ROS Products (arb. units), Asc Radicals (arb. units), NO Radicals (arb. units) and MDA (Mmol/L), GSH-Px (U/ml), SOD (U/ml) and PCC (ng/ml) for Buffaloes in both Seasons (x ± SD)

	Group B1			Group B2		
Indicators	**1**	**1.1**	**1.2**	**2**	**2.1**	**2.2**
	Total	**Pregnant**	**Non-pregnant**	**Total**	**Pregnant**	**Non-pregnant**
n	**16**	**9**	**7**	**8**	**3**	**5**
ROS arb. units	1.98±0.43	1.92±0.47	2.04±0.41	1.73±0.63	1.76±0.63	1.70±0.79
Asc radicals arb. units	0.25±0.17	0.25±0.13	0.25±0.22	0.59±0.11	0.56±0.09	0.63±0.15
NO arb. units	8.55±1.62	8.70±1.71	8.36±1.61	7.87±2.38	6.79±1.61	9.69±2.60
MDA mmol/L	2.44±0.32	2.64±0.35	2.36±0.28	2.49±0.27	2.49±0.35	2.53±0.16
SOD (U/ml)	11.29±1.11	11.31±1.24	11.27±1.01	12.95±1.23	12.67±1.13	13.42±1.12
GSH-Px (U)	56.03±3.96	57.08±3.76	54.67±4.07	57.28±3.42	58.33±3.64	55.55±2.70
PPC ng/ml	2.81±0.83	2.60±0.26	3.09±1.22	2.81±0.56	2.96±0.69	2.57±0.14
Total NO (mM)	49.78±4.64	49.56±4.23	50.05±5.07	47.92±5.39	48.34±5.67	47.21±5.39

Asc radicals: 1-2*;1.1-2.1*;1.2-2.2*(* p< 0.001); NO:1.1-2.1**; 1.2-2.2**(** p< 0.05); SOD: 1-2**, 1.1-2.1**, 1.2-2.2**.

accumulation of ROS and depletion of antioxidant defenses [15]. The first enzyme that contributes to the body's antioxidant defense system is superoxide dismutase. It is believed that the high activity of SOD improves the balance of the antioxidant system and contributes to neutralizing the action of harmful free radicals. From the presented results, we establish high superoxide dismutase activity in buffaloes during the breeding season (winter) compared to the non-breeding season (spring-summer). In support of our results are the recorded values of SOD in a study conducted with buffaloes in Egypt [7]. In pregnant buffaloes, SOD activities are similar in both seasons. Hozyen *et al*. [16] reported similar concentrations of SOD during the year. According to Megahed *et al.* [8] and Sakatani *et al*. [17], during the summer season, due to heat stress, SOD values decrease compared to winter in Egyptian buffaloes and cattle. Chetia *et al*. [18] found lower SOD activity during the breeding season compared to non-breeding in Zebu cattle. During the warmer months, the production of hydrogen peroxide in the animal body may increase, reflecting an increase in the activity of SOD. To neutralize this hydrogen peroxide, glutathione peroxidase, and catalase activity are also increased [19,20]. Thus, a positive and significant relationship exists between GSH-Px, SOD, and CAT activities. These antioxidant enzymes are sensitive markers of oxidative stress, and their levels may increase or decrease in response to reactive oxygen species. Superoxide dismutase, which catalyzes superoxide dismutase, becomes a protective mechanism against oxidative stress. The role of intracellular SOD is to clear superoxide ($\bullet\bar{O}_2$), which is produced by active mechanisms, including several enzyme systems, as part of normal cellular functions [21].

Malondialdehyde is one of several low molecular weight end products formed during the induced radical decomposition of a polyunsaturated fatty acid. MDA readily reacts with thiobarbituric acid to produce a red pigment that can be easily measured by spectrophotometry in the form of reactive substances. Malondialdehyde is a product of lipid peroxidation and therefore changes in its concentrations are used as a biomarker of oxidative stress [4,22]. In our study, MDA values during the non-breeding season were slightly elevated. Hozyen *et al*. [16] observed a higher level of serum MDA in buffalo in the summer compared to the winter season. Higher concentrations may be due to thermal stress in summer compared to the winter season [23]. Similar levels during the summer season were recorded by Lallawmkimi *et al*. [24] in buffaloes and by Bernabucci *et al*. [5] in cows. Kumar *et al*. [7] suggest that buffaloes in Egypt are also affected by oxidative stress in summer, similar to dairy cows [5,17,25]. Glutathione peroxidase is involved in protecting cells against the harmful effects of ROS/RNS, along with superoxide dismutase [26-28].

In general, glutathione peroxidase has similar levels between the summer and winter seasons. This suggests that the season has no pronounced effect on GSH-Px concentration [17]. In contrast, Simonov *et al*. [29] found that cows had higher levels of oxidants in the blood and lower antioxidant activity in winter compared to summer. Total nitric oxide analysis is used to quantify NO in biological fluids. The kit uses the enzyme nitrate reductase to convert nitrate (NO_3) to nitrite (NO_2). Total NO is a key biological indicator that plays an important role in immune responses and apoptosis. Total nitric oxide is also known as "endothelial-derived relaxing factor" or "EDRF", synthesized from L-arginine, oxygen, and NADPH by various NO syntheses. Most of the NO in the cell is oxidized to nitrite and nitrate, and therefore concentrations of these anions are usually a quantitative measure of NO production [30]. Nitric oxide plays an important role in the process of follicular development due to the effect of vasodilation and stimulation of steroidogenesis [31]. The increase in blood flow to the ovaries, particularly to the follicles, is directly related to the increase in NO concentration during the follicular phase [32,33]. In the present study, higher values of total oxide were found in buffaloes during the breeding season (highest levels in pregnant women) than in the non-breeding season. Reactive oxygen species and NO radicals cause peroxidation of polyunsaturated fatty acids, producing α, β-unsaturated aldehydes such as 4-hydroxynonenal (4-HNE), malondialdehyde, and others. These aldehyde by-products of lipid peroxidation are generally accepted markers of oxidative stress and are considered to be the best indicators [34].

CONCLUSION

Comparing the obtained results for oxidative stress and antioxidant activity with respect to pregnancy rates depending on the season showed that pregnancy in buffaloes during the breeding season was realized at higher values of NO and SOD. Increased oxidative stress was observed, resulting in a statistically significant increase in serum ROS products, as well as decreased SOD activity in buffaloes during the

breeding season. The lowest levels of reduced glutathione (GSH-Px) were recorded in non-pregnant animals during both study periods. Total nitric oxide and protein carbonyl content (PPC) were increased in buffaloes during the breeding season.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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