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Oxidative stress profiling in repeat breeder Surti buffalo

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Abstract
Repeat breeding is very important infertility problem in Surti buffaloes caused by various factors which causes low milk production and reduced number of calves per animal leading to economic losses at farmer and industry levels. This study was conducted to study the oxidative stress which is an important factor of repeat breeding in Surti buffalo. The blood samples were collected at Livestock Research Station, NAU, Navsari, Gujarat and analyzed for glucose, BUN, Mg^{2+}, Na^{+}, K^{+}, Ca^{2+} and Cl^{-} in the control and repeat breeder Surti buffalo. These samples were analyzed for various parameters of oxidant/antioxidant status in repeat breeder buffalo. The level of malondialdehyde was significantly increased ($p<0.05$) as compared to normal Surti bufaalo (2.40±0.16 vs. 4.52±0.20). Nitric oxide level was also increased significantly in repeat breeder buffalo as compared to normal buffalo (24.76±1.01 vs. 42.01±0.58). Level of blood urea nitrogen (BUN) was significantly higher in repeat breeder buffalo as compared to normal. Moreover, the level of Glucose, SOD, catalase, glutathione and total oxidant capacity were significantly decreased in the repeat breeder Surti buffalo ($p<0.05$). Therefore, the study concluded that repeat breeder Surti buffalo is under oxidative stress and should be provided adequate amount of antioxidants and minerals in their food.

Keywords: Surti buffalo, oxidative stress, repeat breeder, nitric oxide, infertility

Introduction
Repeat breeding (RB) is a major factor involved in infertility of buffalo. In India the Incidence of repeat breeding varies between 5-32 percent in cows and 6-30 percent in buffaloes (Gupta *et al*., 2005) [12]. Taraphder *et al*., (2002) [32] reported 12.14 percent incidence of repeat breeding in Murrah buffaloes. It is a substantial problem in cattle breeding leading to large economic loss for the dairy producer due to more inseminations, increased calving interval and increased culling rates (Bartlett *et al*., 1986; Lafi *et al*., 1992) [6, 17]. The main constraints of Surti buffalo developments are reproductive disorders, poor nutrition and parasitic infections. Ovarian inactivity, silent heat, endometritis and repeat breeding are the main reproductive disorders in buffaloes (Ahmed *et al*., 2010) [3]. RB may be defined as failure to conceive from three or more regularly spaced services in sub fertile animals and it should not have the anatomical or infectious irregularities (Zemjanis, 1980) [35]. The animals have apparently normal genitalia with clear discharge and having normal oestrous cycle length. The animals fail to become pregnant inspite of timely inseminations with proper technique of insemination. Huge economic losses are encountered due to high incidence (20-39%) of repeat breeding (Nanda and Singh, 2008) [20]. Causes of RB include oxidative stress, estrus detection errors, endocrine dysfunction, ovulatory defects, poor fertilization rates and or early embryonic loss (Nanda and Singh, 2008) [20].

Free radicals are also involved in many infertility related diseases. A free radical is defined as molecular species capable of independent existence and containing one or more unpaired electrons making them paramagnetic and relatively active. These are formed as natural byproducts of oxygen metabolism and serve the purpose of killing bacteria and refuse body matter but when out of control, they become toxic and start damaging body tissues by a process called oxidative stress. There are many causes of oxidative stress that can cost producer money. Diseases, fast growth, early lactation, obesity, adverse environmental conditions such as heat stress increase oxidative stress. Moreover, diet also plays a key role; vitamin and mineral imbalance can be linked to oxidative stress as well. Substances that neutralize the potential ill effects of free radicals are called antioxidants or free radical scavengers. Imbalance between these oxidants and antioxidants is responsible for tissue injury

and affects fertility (Jozwik *et al*., 1999; Sikka, 2001; Shen and Ong, 2000) [13, 30, 29].

Reactive oxygen species (ROS) has a role in pathological processes involving female reproductive tract, whereas it affects multiple physiological processes from oocyte maturation to fertilization, embryo development and pregnancy (Agarwal *et al*., 2003) [2]. It also causes the lipid peroxidation. Nitric oxide has a relaxing effect on smooth muscle and has similar effects on tubular contractility. Increased nitric oxide levels in fallopian tube are cytotoxic to invading microbes and also may be toxic to spermatozoa (Roselli *et al*., 1995) [26]. Superoxide dismutase (SOD) controls steroidogenesis in the ovaries; there is a negative correlation between oxidative stress and ovarian steroidogenesis (Suzuki *et al*., 1999) [31]. And its activity is depleted by lipid peroxidation (Jozwik *et al*., 1999) [13]. Glutathione peroxidase maintains the low levels of hydroperoxide inside the follicle which is important in gametogenesis and fertilization **(**Paszkowski *et al*., 1995) [22]. Increase in the total antioxidant capacity (TAC) was seen in follicular fluid of oocyte that later were successfully fertilized. Lower TAC is predictive of decreased fertilization potential. Keeping the above facts and the importance of RB in Surti buffalo, we decided to measure the level of oxidants and antioxidants in the repeat breeder Surti buffalo.

Materials and Methods

Blood **s**amples were collected from the jugular vein of repeat breeder and fertile Surti buffaloes into anticoagulant vacuotainer tubes as well as clot activator vacuotainer tubes at Livestock Research Station, Navsari Agricultural University, Navsari and clinical camp organized by College of Veterinary Science and Animal Husbdnary, NAU, Navasri under the South Gujarat Heavy Rain fall Agro-climatic Zone. Laboratory investigations were carried out at Department of Veterinary Physiology and Biochemistry, COVS & AH, NAU, Navsari. Plasma and Serum were harvested from the above samples and stored at -20ºC for further analysis. Afterward blood samples were analyzed for nitric oxide, lipid peroxidation, SOD (superoxide dismutase), catalase, glutathione-R and TAC (total antioxidant capacity) at Department of Veterinary Physiology and Biochemistry, COVS & AH, NAU, Navsari.

Estimation of nitric oxide was performed by Modified Griess Reaction Method (Montgomery and Dymock, 1961) [19]. $NO^{\bullet}$ formed or produced is relatively unstable in the presence of molecular oxygen (O_2) and will rapidly and spontaneously auto oxidize in the gas phase to yield NO_2^- and NO_3^-. This method requires that NO_3^- first be reduced to NO_2^- by Cd-Cu alloy and then the total NO_2^- is determined by the Griess reaction. The Griess reaction involves the formation of a chromophore during the reaction of NO_2^- with sulfanilamide and heterocyclic amine such as N-1-α-naphthyl ethylene diamine dihydrochloride (NEDA) under conditions of low pH. During this reaction acidified NO_2^- undergoes diazotization with sulfanilamide to form a diazonium salt. The diazonium salt then couples to NEDA to form a purple compound that has absorption maximum at 545 nm. On the contrary, NO_3^- does not react with Griess reagents and therefore must be reduced quantitatively to NO_2^- by Cd-Cu alloy or NADPH reductase to react with Griess reagents. Lipid peroxidation was estimated by the method described by Alvarez and Storey, 1982 [4]. Lipid proxidation process generates MDA as one of the breakdown products. Thiobarbituric acid (TBA) is used in this method to measure MDA. MDA form a 1:2 adduct with the thiobarbituric acid and produces the TBA-MDA complex, which gives a light pink colour, which can be read by spectrophotometer at 534 to 570 nm. In this assay, an MDA standard is used to construct a standard curve against which unknown samples can be plotted. Estimation of catalase was performed by Aebi, 1984 method. In the ultraviolet range H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance (ΔA_{240}) per unit time is a measure of the catalase activity. To avoid inactivation of the enzyme during the assay (usually 30 sec) or formation of bubbles in the cuvette due to the liberation of O_2, it is necessary to use a relatively low H_2O_2 concentration (10 mM). The H_2O_2 concentration is critical in as much as there is direct proportionality between the substrate concentration and the rate of decomposition. Due to the special situation in catalase the dependence of the H_2O_2 decomposition on the temperature is small so that measurements can be carried out between 0 and 37°C; however, 20°C is recommended. The pH activity curve relative to V0 has a fairly broad pH optimum (pH 6.8-7.5), so measurements are made at pH 7.0. Estimation of SOD was performed by Misra and Fridovich, 1972 [18] method. Total antioxidant capacity was measured by the FRAP (ferric reducing antioxidant power) method (Benzie and Strain, 1996) [7]. The FRAP (ferric reducing antioxidant power) method relies on the reduction by the antioxidants, of the complex ferric ion-TPTZ (2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine). The binding of Fe^{2+} to the ligand creates a very intense navy blue color. The absorbance can be measured to test the amount of iron reduced and can be correlated with the amount of antioxidants. Ascorbic acid was used as references. Data were analyzed using one way analysis of variance (ANOVA) to compare means between the two groups. Results were expressed as Mean± standard deviation (SD). All statistical analysis was performed using Statistical Program for Social Science (SPSS) version 16 (IBM/Chicago, IL, USA). Probability $p<0.05$ was considered statistically significant.

Result and Discussion

Level of calcium in repeat breeder Surti buffalo

The mean serum calcium level in repeat breeder Surti buffalo was reduced from 1.208±0.02 to 0.886±0.02 (Table-2). Similarly, the mean serum calcium level in anestrous cattle was significantly lower. These values, however, did not differ significantly between repeat breeder (7.82±0.06 mg/dl) and anestrous (7.31±0.02) cattle. The serum calcium level was also found to be non-significantly higher in normal cyclic cattle than in repeat breeder cattle (Aslam and Tucker, 1998) [5]. The decrease in calcium levels in blood serum of anestrous and repeat breeder cattle could be caused by pathogens or the negative effects of increased reactive oxygen species, which damage the junctional complex in blood vascular channels or disrupt normal homeostatic mechanisms, resulting in poor absorption or increased losses from blood. Previously, similar results were obtained in anoestrous, repeat breeder, and normal cyclic Sahiwal cows (Aslam and Tucker, 1998) [5].

Level of glucose in repeat breeder Surti buffalo

In repeat breeder Surti buffalo the blood glucose levels was found to lower than the normal cycling buffalo (1.2080.02 to

0.8860.02 mmol/L). Similarly, repeated breeding cows have lower blood glucose levels than normal cycling cows (Prihatno *et al*. 2013) [24]. According to Khan *et al*. (2010) [15], blood glucose levels in repeated breeding dairy cows were lower than in normal dairy cows, with the highest blood glucose level in repeated breeding being 50.351±3.54 mg/dl and 63.41±4.87 mg/dl in normal cycling dairy cows. The homeostatic mechanism of the insulin hormone keeps blood plasma glucose concentration relatively constant (Setiadi *et al*., 2003) [28]. Several factors, including energy content, carbohydrate type, and the role of the insulin hormone, influenced blood glucose levels. Dutta *et al*. (1988) [10] discovered that an-oestrus animals had significantly lower serum glucose levels than normally cycling animals. Reduced blood glucose levels in repeat breeder cows in the current study could be due to either instability between hepatic output and peripheral glucose uptake or defects in the endocrine regulatory mechanisms that influence these processes. Glucose levels may be affected by abnormal hormone-producing organ function. Reduced glucose levels influenced the pituitary hypothalamus, which controls reproductive function, resulting in inhibition of Gonadotropin-releasing hormone-regulated growth and follicular development, ovulation, and maintaining an appropriate uterine environment for embryonic development (Prihatno and Gustari, 2003) [25]. Less glucose levels affected the pituitary hypothalamus, which controls reproductive function, resulting in inhibition of Gonadotropin-releasing hormone-regulated growth and follicular development, ovulation, and maintaining an appropriate uterine environment for embryonic development (Prihatno and Gustari, 2003) [25].

Level of blood urea nitrogen in repeat breeder Surti buffalo

The BUN level in repeat breeder Surti buffalo is significantly higher than the normal cycling buffao from 37.33±0448 to 23.44±1.819 mg/dl (Table-2). The blood urea level in repeat breeding FHG cows was 31.99±4.80 mg/dl, while it was 28.10±3.97 mg/dl in normal cycling cows. Pregnancy rates were reduced when BUN levels were high (Butler, 2005) [8]. A high urea concentration was linked to fertility problems, decreased available energy, environmental pollution, and economic losses (Setiadi *et al*., 2003) [28]. Increased blood urea nitrogen levels tend to alter ovarian and uterine physiology, and uterine environment changes, such as decreased pH during the luteal phase, may play a role in reducing fertility. Increased plasma urea and ammonia concentrations in cows have a negative effect on the quality of uterine fluid due to increased ammonia concentrations, which along with urea; have direct and toxic effects on the endometrium and a negative effect on the mRNA expression levels of endometrial fertility-related genes. High ammonia content in uterine fluid is caused by amino acid metabolism, specifically the use of glutamine for energy. Ammonia entering the uterine lumen increased the luteal phase and exacerbated the negative effects of urea on pH and other secretions, resulting in decreased fertility. Urea that circulates in blood vessels can be measured as urea nitrogen in a fraction of blood plasma or serum and is commonly expressed as the BUN value. A previous study found that a high urea concentration was linked to decreased fertility, less available energy, pollution, and economic losses. High levels of urea in the blood may enter the uterine reproductive tract and affect uterine pH, altering uterine micro-conditions and decreasing fertility rates, ultimately leading to an increased likelihood of repeat breeding.

Level of electrolytes in repeat breeder Surti buffalo

The level of Mg^{2+} and Na^{+} was found to be lower in repeat breeder Surti buffalo as compared to the normal cyclic buffalo. There was no significant difference in Cl^{-} level in both the repeat breeder Surti buffalo and normal cycling buffalo whereas the K^{+} concentration in repeat breeder Surti buffalo is about double than the normal cyclic Surti buffaloes (Table-2).

Level of LPO in repeat breeder Surti buffalo

The level of malondialdehyde was found to be significantly higher in repeat breeder Surti buffalo (Table-1) as compared to the normal cyclic buffalo (2.403±0.169 vs 4.522±0.203). The increased level of serum MDA could be due to the increased production of free radicals (ROS) during decrease in antioxidant defence and various infections (Turk *et al*., 2013) [16]. The free radicals released by phagocytes react with DNA, lipids, and various other macromolecules such as proteins, which act as natural targets of oxidation and alter the normal functions of proteins, malfunctioning of enzymes, and amino acids present in different enzymes results in oxidative damage (Poulsen, 2005) [23]. The increased levels of serum MDA and NO in present study could be due to the inflammatory process induced by different pathogens and are important biomarkers of inflammatory disease (Kullisaar *et al*., 2013) [16].

Level of Nitric oxide in repeat breeder Surti buffalo

The level of nitric oxide was found to be significantly higher in repeat breeder Surti buffalo (Table-1) as compared to the normal cyclic buffalo (42.01±0.587 vs 24.76±1.005). NO relaxes smooth muscles and has a similar effect on tubular contractility. An abnormal NO concentration can cause tubal motility dysfunction, resulting in ovum retention, delayed sperm transport, and infertility. Increased NO levels in the fallopian tubes, on the other hand, have been reported to be cytotoxic to invading microbes and potentially toxic to spermatozoa (Agarwal *et al*., 2003) [2]. Furthermore, Seino *et al*. (2002) discovered that NO inhibits ovarian steroid genesis. On the other hand, it has been reported that increased NO levels in the fallopian tubes are cytotoxic to invading microbes and may also be toxic to spermatozoa. Furthermore, NO inhibits ovarian steroid genesis, (Seino *et al*., 2002) [27]. Endothelial NO synthase has been found in corpora lutea and expressed in the mid and early luteal phases, as well as to a lesser extent in the late luteal phase. Furthermore, NO inhibits steroid genesis in the corpus luteum and has luteolytic activity mediated by increased prostaglandins and apoptosis (Vega *et al*., 2000; Friden *et al*., 2000) [34, 11].

Level of SOD in repeat breeder Surti buffalo

The level of SOD was found to be significantly lower in repeat breeder Surti buffalo (Table-1) as compared to the normal cyclic buffalo (301.86±3.75a vs 350.24±4.76). SOD is present in the ovarian tissue and it was found that there is a correlation between SOD and Ad4BP which is a steroidogenic transcription factor that induces transcription of the steroidogenic P450 enzyme. Thus, it controls steroid genesis in the ovaries. The correlation between Ad4BP and SOD expression suggests an association between oxidative stress and ovarian steroidogeneis (Suzuki *et al*., 1999) [31]. The

preovulatory follicle has a potent antioxidant defense, which is depleted by the intense peroxidation (Jozwik *et al*. 1999) [13].

Level of Glutathione-reduced in repeat breeder Surti buffalo

Glutathione-Reduced was found to be significantly lower in repeat breeder Surti buffalo (Table-1) as compared to the normal cyclic buffalo (2.068±0.199 a vs 4.464±0.116). Glutathione peroxidase may also play an important role in gametogenesis and fertilization by maintaining low levels of hydroperoxides inside the follicle (Paszkowski *et al*., 1995) [22]. Meanwhile, De Matos and Furnus, (2000) [9] discovered that glutathione is present in the oocyte and tubal fluid and helps the zygote develop beyond the 2-cell block to the morula or blastocyst stage.

Level of Total Antioxidant capacity in repeat breeder Surti buffalo

Total antioxidant capacity was found to be significantly lower in repeat breeder Surti buffalo (Table-1) as compared to the normal cyclic buffalo (0.962±0.064 a vs 1.936±0.070). TAC levels increased in the follicular fluid of oocytes that were successfully fertilized. As a result, lower TAC predicts lower fertilization potential (Oyawoye *et al*., 2003) [21].

Table 1: Status of Oxidant and antioxidant level in repeat breeder Surti buffalo

Oxidant/ antioxidant	Normal buffalo (N=10)	Repeat breeder buffao (N=10)
Malondialdehyde (nmol/l)	2.403±0.169	4.522±0.203[a]
Nitric oxide (µmol/l)	24.76±1.005	42.01±0.587 [a]
SOD (U/ml)	350.24±4.76	301.86±3.75 [a]
Catalase (kU/l)	3.23±0.065	1.95±0.049 [a]
Glutathione-R (µmol/l)	4.464±0.116	2.068±0.199 [a]
TAC (mmol/l)	1.936±0.070	0.962±0.064 [a]

Values are the mean ± SEM of three different samples. Superscript [a,] indicate significant differences (p<0.05).

Table 2: Level of electrolytes, Glucose and BUN in repeat breeder Surti buffalo

Parameters	Control samples	Repeat Breeder
Na^+	137.8±0.7621mmol/L	136.4±0.690 mmol/L
K^+	4.378±0.094 mmol/L	8.783±0.060 mmol/L
Cl^-	102.0±0.57 mmol/L	102.2±0.814 mmol/L
Ca^{2+}	1.208±0.020 mmol/L	0.8867±0.020 mmol/L
Mg^{2+}	0.835±0.033 mmol/L	0.068±0.0173 mmol/L
Glu	35.00±1.472 mg/dL	22.61±1.028 mgl/dL
BUN	23.44±1.819 mg/dL	37.33±0.448 mg/dL

Values are the mean ± SEM of the biochemical parameters in different samples.

Conclusion

Oxidant/antioxidant status is very important determining factor in the fertility in Surti buffalo. The level of malondialdehyde and nitric oxide were increased significantly (p< 0.05) in RB Surti buffalo compared to normal cyclic buffalo. Level of blood urea nitrogen (BUN) was also significantly higher in repeat breeder buffalo. However, the level of Glucose, SOD, catalase, glutathione and total oxidant capacity were significantly decreased in the repeat breeder Surti buffalo. Therefore, it is concluded that the repeat breeder Surti buffalo is under oxidative stress and should be provided adequate amount of antioxidants and minerals in their food.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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