Unraveling the Oxidative stress: Study on biomarkers and their gene expression in periparturient Ongole Cattle

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Abstract

This study investigates the oxidative stress markers in periparturient Ongole cows, a dual purpose Indigenous breed from Andhra Pradesh, India. Livestock significantly contributes to India's economy and understanding stress responses during the critical transition period from late pregnancy to early lactation is crucial. During this period, cows experience negative energy balance and increased production of reactive oxygen species (ROS), leading to oxidative stress (OS) that disrupt vital functions. However, the antioxidant defence system specific for every species quenches the free radicals and confers protection against the adverse effects of oxidative stress. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH) and glutathione reductase (GSH) are the major antioxidant enzymes directly involved in defence mechanism. In view of their significance in native breeds of cattle like Ongole, the present study focused on the biochemical assays and qPCR studies of these antioxidant enzymes. The results revealed significant changes in oxidative stress parameters, providing insight into the stress adaptation mechanisms of Ongole cows during the transitional period. From the present study, it was concluded that, the day zero of parturition was found to be more stressful to the animals when compared to antepartum and postpartum periods. The reduced levels as well down regulation of mRNA of CAT,SOD, GPx from prepartum to day 0 of calving and increased trend of GSH,MDA particularly towards one-week prepartum indicated that the animals competently alleviated the stress of transition.

Keywords: Ongole cattle; periparturient period; oxidative stress

Introduction

Livestock is a vital component of the Indian economy, contributing 4.11% to the total GDP and 25.6% to the agricultural GDP, with India hosting the largest livestock population globally (20th Livestock Census). Among the indigenous breeds, Ongole cattle, native to the coastal districts of Andhra Pradesh, are renowned for their dual-purpose utility in draft power and moderate milk production (Priyanka *et al.*, 2024). The periparturient period from late pregnancy to early lactation is marked by negative energy balance and increased production of ROS, leading to oxidative stress (Singh *et al.*, 2017; Abdelrazek *et al.*, 2018 and Vasantha *et al.*, 2024). This stress can disrupt crucial physiological and metabolic functions (Grummer *et al.*, 2004); however it is overcome by a complex antioxidant defense system that is inherent to every species. Antioxidants function as radical scavengers, electron donors, peroxide decomposes, hydrogen donors, enzyme inhibitors, metal ion chelating agents, singlet oxygen quenchers, co-antioxidants, gene expression regulators (Lobo *et al.*, 2010 and Vasantha, 2024). They work at several levels of defence, including preventive, radical scavenging, repair, de novo, and adaptation. The first line of defence is preventive antioxidants, which inhibit free radical formation by reducing hydro peroxides and hydrogen peroxide to alcohols and water without producing free radicals. SOD, CAT, GPx, and glutathione reductase (GSH) are the major antioxidant enzymes directly involved in defence mechanism (Pham Huy *et al.*, 2008). CAT shields the cell from damage caused by the secondary generation of highly reactive hydroxyl groups from superoxide ion to H_2O_2. SOD catalyses the breakdown of super oxide anion into oxygen and hydrogen peroxide (Zelko *et al.*, 2002). GPx is a detoxification enzyme that catalyses the breakdown of hydrogen peroxide and organic hyperperoxides. It functions as an important intracellular antioxidant, protecting membrane lipids, proteins, and nucleic acids from ROS mediated damage. Malondialdehyde (MDA) is one of the several low molecular weight end product formed during the free radical induced decomposition of polyunsaturated fatty acid. Lipid peroxidation is a non-enzymatic chain reaction based on oxidation of unsaturated fatty acids and important consequence of OS (Konvicna *et al.*, 2015). Reactive aldehydes such as malondialdehyde and 4-hydroxynonenal are produced by lipid peroxidation (Puppel *et al*., 2015).

Despite the significant roles of these biomarkers as mentioned above, studies on oxidative stress markers in Ongole cows were limited. Hence, it is essential to understand the expression of oxidative stress biomarkers which are involved in major antioxidant defence mechanisms like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH) and malondialdehyde (MDA) through both biochemical assays and qPCR studies (Pham Huy *et al*., 2008).

Materials and methods

The present study was conducted on Ongole cattle at the Livestock Research Station (LRS), Lam, Guntur, Andhra Pradesh, which is located at 16.3067° N latitude and 80.4365° E longitude, at an elevation of 33 meters above mean sea level from June-August, 2023. Twelve adult Ongole cattle, comprising six pregnant (treatment group) and six non-pregnant (control group), were selected for the study. These 2 to 3-year-old cattle were housed in a loose housing system, with ad libitum access to feed and water, and were maintained under regular deworming and vaccination schedules. The enzymatic assays and gene expression analysis was carried out in the Department of Veterinary Physiology, NTRCVSc, Gannavaram.

Blood samples were collected weekly from the jugular vein, with 10 ml of whole blood collected in EDTA-coated vacutainers for oxidative stress biomarker analysis and 2 ml in plain vacutainers for PBMC separation and RNA isolation. The study employed various biochemical assays, enzymatic activity assays for SOD (Madesh and Balasubramanian, 1998), CAT, GPx (Paglia and Valentine, 1967), reduced GSH (Ellman, 1959) and malondialdehyde (Balasubramanian et al., 1988).

PBMCs were isolated using Histopaque-1077(Sigma, USA) and RNA was extracted using TRIZOL reagent (Manjari *et al.*, 2015). RNA quality and quantity were assessed using a Nanodrop spectrophotometer (Nanodrop, ND-1000, Thermo Scientific, USA) and cDNA synthesis was performed using a BIO-RAD iScript Select cDNA Synthesis kit. Primers for target genes were designed using Primer3 Plus softwarewith the nucleotide sequence of the gene available in the Gene bank database at (www.ncbi.nim.nih.gov) (Table.1), and the synthesized cDNA was used for quantitative PCR analysis to study gene expression. The end product obtained from the qPCR assay was subjected to agarose gel electrophoresis and was validated by using DNA markers.

The results were represented as Mean±SE and analyzed using GraphPad PRISM 9.0. Relative gene expression was calculated using the $2^{-\Delta\Delta}$Cq method, with significant differences between groups determined by one-way ANOVA and Duncan's multiple range test, and comparisons between control and periparturient periods conducted via independent sample T-test. Statistical significance was set at $\alpha<0.05$.

Results

Oxidative Stress Markers

The mean ±SE of SOD, CAT, MDA, GPX and GSH in periparturient Ongole cows on 3 weeks prepartum, 2 weeks prepartum, 1 week prepartum, day zero, 1st, 2nd and 3rd week postpartum were mentioned in

Table 1: Primer sequence of the genes

Genes	Primer sequence from 5' to 3'	Size (bp)
SOD	F: CACAAGGCTGTACCAGTGC R:CCACAATGGCAACACCGTTT	132
CAT	F: CCCACGAAGACCCTGACTAC R: GTCATTGTGAGGCCAAACCT	161
GPX	F: TTCCCTTGCAACCAGTTTGG R: TCATCCATTTCCACAGAGGGT	145
	Reference gene	
GAPDH	F: AAAGTGGACATCGTCGCCAT R: CCGTTCTCTGCCTTGACTGT	144

Table 2: Oxidant and anti oxidant parameters during the transitional period in ongole cows (Mean±SE, n=6)

Parameters	**Average**	Stage of transition period						
		-3 week	**-2 week**	**-1 week**	**Day 0**	**+1 Week**	**+2 Week**	**+3 week**
SOD (U/mg of protein)	3.44^{NS}±0.23	4.06^{a}±0.50	3.85^{a}±0.45	3.59^{a}±0.35	3.79^{a}±0.49	2.71^{a}±0.62	2.61^{a}±0.62	3.47^{a}±0.72
Catalase	116.75^{**}±20.49	141.75^{a}±24.39	120.02^{ab}±26.46	80.80^{b}±18.99	92.91^{b}±16.45	114.52^{ab}±26.23	138.00^{ab}±31.08	130.09^{ab}±23.27
MDA(µmoles)	3.68^{NS}±0.14	3.72^{a}±0.31	3.86^{a}±0.54	3.13^{a}±0.22	3.35^{a}±0.14	4.78^{a}±0.86	3.41^{a}±0.37	3.54^{a}±0.35
GPX(u/ml)	38.95^{*}±4.22	45.09^{ab}±9.89	37.31^{ab}±8.59	35.59^{ab}±2.54	35.31^{b}±7.53	27.12^{b}±4.52	46.12^{a}±11.90	46.09^{a}±9.36
GSH	3.71^{**}±0.10	4.36^{ab}±0.36	3.67^{bc}±0.16	1.39^{d}±0.07	3.24^{c}±0.16	3.96^{bc}±0.24	4.18^{ab}±0.31	5.16^{a}±0.24

The Mean with same superscript (a,b, c) in a column do not differ significantly at 5% level of significance

Table 3: Independent sample t test of oxidant and anti oxidant parameters during the transitional period and control in ongole cows (Mean±SE, n=6)

Parameters	**Control**	**Stage of transition period**		
		-3 week	**Day 0**	**+3 week**
SOD (U/mg of protein)	3.61±1.16	4.06^{NS}±0.50	3.79^{NS}±0.49	3.47^{NS}±0.72
Catalase	91.19±47.13	141.75^{NS}±24.39	92.91^{NS}±16.45	130.09^{NS}±23.27
MDA(µmoles)	3.03±0.41	3.72^{NS}±0.31	3.35^{NS}±0.14	3.54^{NS}±0.35
GPX(u/ml)	36.45±14.90	45.09^{NS}±9.89	35.31^{NS}±7.53	46.09^{NS}±9.36
GSH	3.89±1.05	4.36^{NS}±0.36	3.24^{NS}±0.16	5.16*±0.24

Table 2 and the mean ±SE SOD, CAT, MDA, GP_X and GSH in control, 3 weeks prepartum, day zero and 3 weeks postpartum periparturient Ongole cows were mentioned in Table 3.

The mRNA expression levels of SOD, CAT and GPx were analyzed in PBMCs collected from periparturient Ongole cows. Mean±SE values of ΔCq and ΔΔCq for SOD, CAT and GPx across various stages (3 weeks, 2 weeks prepartum, 1 week prepartum, day zero, 1st, 2nd, and 3rd weeks postpartum) are provided in Figure nos. 1-3. The qPCR product amplicon size was visualized on a 2% agarose gel, with amplification plots and melt curves shown from Figure nos. 4-11.

Discussion

Dairy cattle are more vulnerable to a variety of metabolic and contagious diseases during the transition period compared with peak lactation (Sordillo *et al*., 2007; Sharma *et al*., 2011). A number of studies reported that oxidative stress is a threat to transition period and increased levels of the same might lead to complications associated with calving (Orhan *et al*., 2003; Castillo *et al*., 2005; Dimri *et al*., 2010; Nasirpour *et al*., 2017; Nachare *et al.*, 2024). A lot of diseases are now regarded at least partly, to be caused by oxidative damages. The nucleic acids, proteins, and lipids are the cell's organic components that are chemically damaged by ROS (Martinelli *et al*., 2021). The functions of the membranes will be disturbed as well the integrity of the membrane will be compromised, causing unwanted enzyme leakage, electrolyte dispersion, metals, and small molecules movement (Behairy *et al*., 2020; Nemec *et al*., 2021). Hence to understand the extent of antioxidant defence offered by the animals against oxidative damage, it is important to estimate oxidative stress markers across the different stages of periparturient period.

Superoxide Dismutase (SOD)

The results of the present study indicated that no significant difference was observed in the levels of SOD during different weeks of the transition period. Similarly, the mRNA expression of SOD during different weeks of the transition period was also non significant. Similarly, no significant difference in SOD levels was observed among prepartum transition period, 0 day of calving and postpartum transition period in comparison to the control.

Although no significant difference was observed in the different weeks of transition period, a decreasing trend from 3 weeks prepartum to 2 weeks postpartum could be observed.

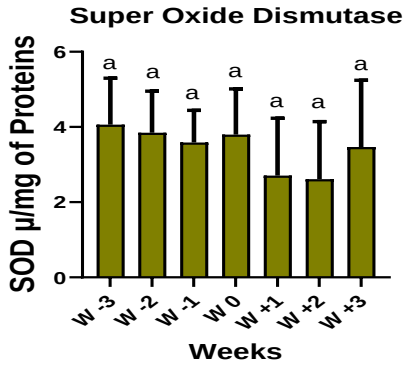

Figure 1: superoxide dismutase (SOD) activity of Ongole cattle during periparturient period

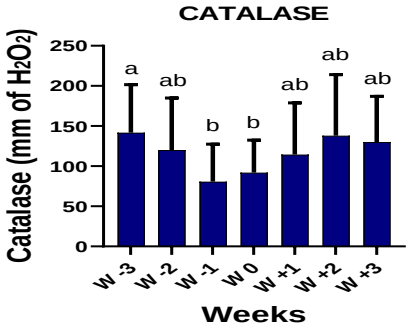

Figure 2: Catalase activity on Ongole cattle during periparturient period

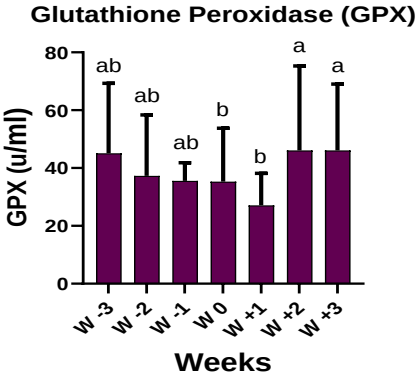

Figure 3: GPx activity in Ongole cattle during periparturient period

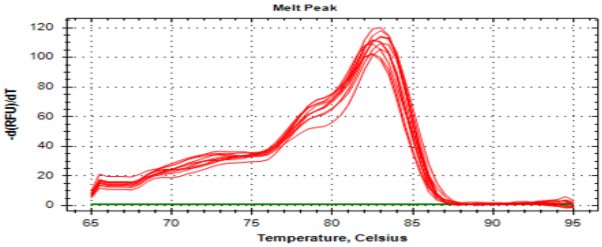

Figure 4: Melt curve of SOD

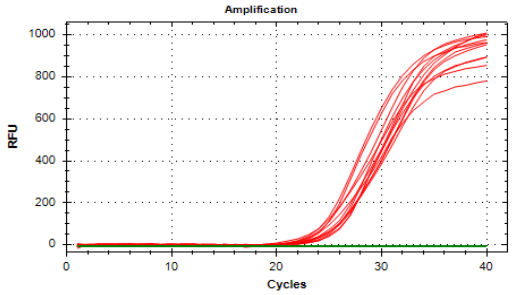

Figure 5: Amplification plot of SOD

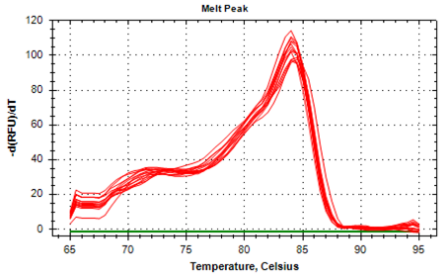

Figure 6: Melt curve of Catalase

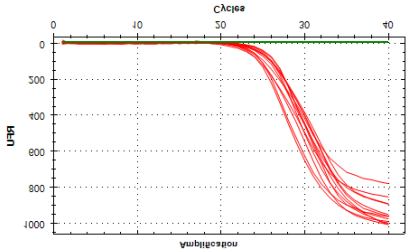

Figure 7: Amplification plot of catalase

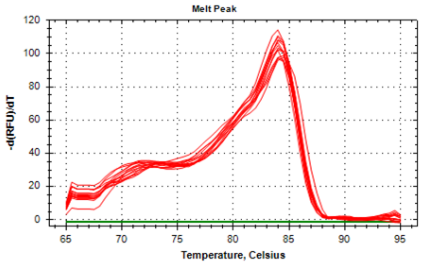

Figure 8: Melt curve of GP_X

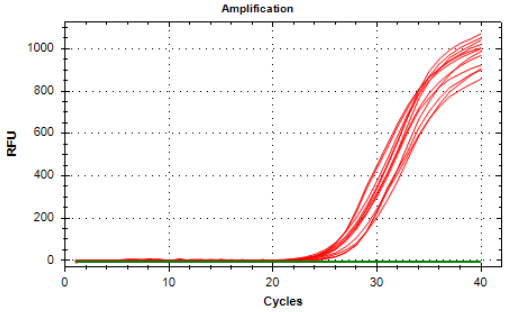

Figure 9: Amplification plot of GP_X

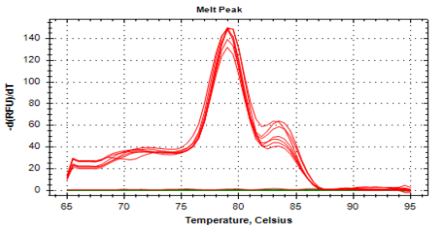

Figure 10: Melt curve of GAPDH

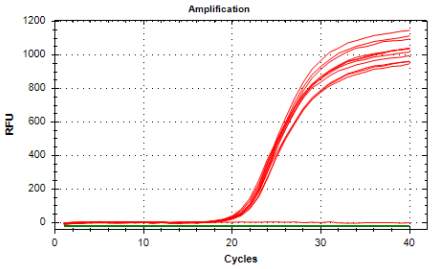

Figure 11: Amplification plot of GAPDH

Catalase (CAT)

In the present study, the CAT activity was found to be significantly higher ($p<0.05$) in 3rd week of prepartum and significantly lower ($p<0.05$) in 1st week of prepartum and day 0 of parturition, while the CAT activity the difference was non-significant between 2nd week prepartum and during postpartum transition period. Similarly, non-significant difference was observed among 3 weeks prepartum, 0 day and 3 weeks postpartum with control animals. The mRNA expression of CAT was upregulated ($p<0.05$) at 3 weeks prepartum and downregulated ($p<0.05$) at one week prepartum and day 0 of parturition.

Similar results were reported by Chandra and Aggarwal (2009) in crossbred cows. The observed pattern of decreased CAT activity from 3 weeks prepartum to day 0 of calving in the present study is indicative of the body's response to overcome the probable oxidative stress during pregnancy. The activated antioxidant enzyme defense system from three weeks prepartum, eliminated ROS by detoxifying H_2O_2. As no antioxidants were supplemented, the antioxidant reserves were exhausted and reflected as significantly lower CAT activity on day 0 of calving. These observations of the present study were in acceptance with the findings of Sihag *et al*., (2021) who reported heat stress and its effect on CAT in buffalo calves of arid climate. As reported by Singh *et al*., (2014), increased CAT activity at three weeks prepartum might be a preparatory mechanism adapted by the animals.

Glutathione Peroxidase (GPx)

The results of the present study indicated that GPx levels were significantly higher ($p<0.05$) on 2 and 3 weeks postpartum; significantly lower ($p<0.05$) on day zero and 1 week postpartum compared to all the other weeks of transition period. The GPx levels among 3 weeks of prepartum, day 0 and 3 weeks of postpartum transition period were found to be non-significant. While in comparison of 3 weeks prepartum, day 0 and 3 weeks postpartum with control no significant difference was observed. The mRNA expression of GPx was upregulated ($p<0.05$) at 2 and 3 weeks postpartum; downregulated ($p<0.05$)on day zero and 1 week postpartum compared to all the weeks of periparturient period.

The significantly higher GPx activity on 2 and 3 weeks postpartum in the present study was found to be similar to Adela *et al*., (2006), where GPx activity in dairy cows was higher in 6th week than 1st week after parturition and suggested that the dairy cows were under oxidative stress close to parturition and early lactation compared to late lactation. The pattern of GPx indicated that the levels of GPx decreased significantly from 3 weeks prepartum to day 0 of calving. The results of the present study were in line with those of Bernabucci *et al*., (2002) in dairy cows. The decreased enzyme activity prepartum might be due to increased reactive oxygen metabolites levels, oxidative stress and lipid peroxidation.

Higher GPx activity similar to that of CAT observed in our study, might be a preparatory activity in late gestation as the energy requirement of the animal changes thereby increasing the production of H_2O_2 by enhanced activity of SOD as observed in the present study concluding;GPx to be a sensitive marker of oxidative stress.

Reduced Glutathione (GSH)

The results of the present study indicated that GSH levels were significantly lower ($p<0.05$) during one week prepartum and significantly higher during three weeks postpartum. The GSH levels decreased towards parturition and then increased postpartum upto three weeks. The GSH levels at three weeks postpartum were highly significant ($p<0.05$) as compared to the control. The results of the present study were in agreement with the findings of Singh *et al*.,(2017) in buffaloes, Sharma *et al*., (2011) in cattle and Vasantha *et al*., (2024) in periparturient Murrah

buffaloes. The pattern in enzyme activity observed from our study might be due to the metabolic demand on the buffaloes for colostrum production during onset of lactation which exceeds the demands of the foetus as a result of which the GSH was reduced in early lactation as also proposed by Sharma *et al*.,(2011), who conducted a trail in crossbred dairy cows and concluded that dairy cows were under more oxidative stress with low antioxidant defense during early lactation or just after parturition than advanced pregnant cows and also attributed that early lactation stage of the dairy cows, with least antioxidant defense were more prone to production diseases like metritis, mastitis and retention of fetal membranes.

Malondialdehyde (MDA)

The results of the present study indicated that no significant difference was observed among the different weeks of transition period. However, although non-significant, there is an increased pattern of MDA upto one week postpartum. In a number of reported studies, it has been observed that MDA levels were significantly higher on day zero of parturition. The increased concentration of MDA is indicative of the level of metabolic stress at parturition and reflected the metabolic adaptation of the animal. Further, after one week postpartum, the animals were relieved from stress and it was associated with a gradual decrease in the level of lipid peroxides that declined steadily as stated (Castillo *et al.,* 2005 and Pathan *et al.,* 2010). The decreasing trend in MDA levels observed from 1 week postpartum to 3 weeks postpartum in our study might be due to the gradual adaptation of the animal's body to the metabolic alterations leading to the animal regaining homeorhesis (Abuelo *et al.*, 2015; Vasantha *et al*., 2024).

No significant difference in MDA levels among 3 weeks prepartum, day zero and three weeks postpartum with control was observed. This non-significance in the MDA values through different stages of the transition may be attributed to the robust adaptation of the Ongole cows to the physiological processes that would not reflect in the antioxidant and/or oxidative stress biomarkers' levels to show significance variation. Also, Ongole breed is not a heavy milch breed due to which there might not be increased lactational to shows significance variation in the MDA levels between different stages of transition period as well with the control group.

Conclusion

From the present study, it was concluded that, the day zero of parturition was found to be more stressful to the animals when compared to ante partum and postpartum periods. Increased levels of CAT, SOD and GPx were indicative of active antioxidant defence mechanism. The down regulation in the expression of CAT, SOD, GPx from 3 weeks prepartum to day 0 of calving ($p<0.05$) further strengthened the fact, the animals competently alleviated the peripartum stress. Hence, CAT, SOD and GPx could be used as stress biomarkers in Ongole cows to determine onset of any post parturient metabolic diseases or complications.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: All experimental procedures were conducted following ethical guidelines approved by the Institutional Animal Ethics Committee.

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