Indian Journal of Animal Sciences **83** (7): 717–724, July 2013/Article

Gender and age bias in immune competence and oxidative stress markers in Murrah buffaloes

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Received: 7 October 2012; Accepted: 27 February 2013

ABSTRACT

Present study was carried out in Murrah buffaloes to recognise variability in basic circulatory levels of various immune response mediators or oxidative stress markers between sexes, ages and estrous cycle stages. Lymphocyte proliferation and plasma IgG varied between age groups and stages of estrous cycle. Lymphocyte proliferation was negatively correlated whereas plasma IgG levels were positively correlated with age. Plasma nitric oxide and tumor necrosis factor α was sex biased. Significantly low levels of NO and TNFα were recorded in diestrous than estrous buffaloes. Xanthine oxidase did not differ between sexes or stages of reproductive cycle. Total leukocytes were higher in prepubertal and at estrus as compared to post pubertal and at diestrous buffaloes. TBARS registered increase with age and was predominant in females at diestrous. Antioxidant power was high in females than males but low in post-pubertal buffaloes. Present findings make out the apparent role of age and sex steroids in immune competence.

Key words: Buffaloes, Gender bias, Nitric oxide, TBARS, TNFα, Xanthine oxidase

Buffalo is an economically important livestock species in Asian and Mediterranean countries with India having 56% of total world buffalo population. Buffaloes in India serve as draught, milk and meat animal, which holds the greatest promise and potential for production (Economic Survey Govternment of India 2008–2009).Sex hormones are being realised as integral signalling modulators of mammalian immune system whose complex interplay maintains homeostasis (Charles 1984) and play a role in the aetiology and course of chronic inflammatory diseases (Cutolo and Wilder 2000). The epidemiological studies reveal age and sex bias in the prevalence of many bacterial and parasitic diseases in domestic animals (Rajput *et al.* 2005, Sinha *et al*. 2006, Upadhyay *et al.* 2007, Ramzan *et al.* 2009). Pampori and Sujata (2012) showed varied effects of sex steroids on cellular immunity *in vitro*. Estrogen and progesterone were reported to affect the function and concentration of polymorphonuclear leukocytes (PMNL) and immunoglobulins in cattle (Roth *et al*. 1983, Lander *et al*. 1990). Sartin *et al*. (2003) showed that estrogen plus progesterone implants favourably alter the time course or disease severity of many of clinical manifestations associated with coccidiosis and endotoxemia in calves.

Lymphocyte proliferation induced by a mitogen/antigen *in-vitro* has been claimed to be representative of cellular immunocompetence (Kristensson *et al.* 1994). Similarly the most abundant immunoglobulin Ig G provides majority of the antibody based immunity (Pier *et al.* 2004). Stuehr and Nathan (1989) have shown nitric oxide (NO) to be a powerful cytotoxic agent for a variety of target cells that was partly regulated by sex steroids. Tumor necrosis factor α (TNFα), a proinflammatory cytokine produced by immune cells and xanthine oxidase (XO) a ubiquitous enzyme, induces a number of different biological effects in various cells, regulating the immune responses (Bemelmans *et al.* 1996, and Harrison 2002). All these parameters are associated with the immune competence, flare and outcome of diseases, therefore, their circulatory profile could provide a sketch of the immune-competence of an animal. The present study could offer guiding principle while approaching for an effective application of immunomodulatory and therapeutic agents in the management of livestock disorders. The use of thiobarbituric acid reactive substances (TBARS) and ferric reducing ability of plasma (FRAP), measures of oxidant-antioxidant balance, could provide complementary information about the homeostasis of the animal. Present study was therefore, aimed at assessing age and gender bias

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in immune response mediators and oxidative stress markers in Murrah buffaloes of pre- and post-pubertal age.

MATERIALS AND METHODS

Healthy Murrah buffaloes (44) of either sex (male and female) were selected from NDRI herd for conducting present study. All these buffaloes were maintained under routine managemental practices as followed for the herd at the institute. Male buffaloes (18) were divided into 3 groups of 6 each on the basis of their age, viz. group 1 (11–13 months); group 2 (22–24 months) and group 3 (34–36 months). Similarly 18 female buffaloes were grouped exactly the same way as in males with group 3 having cyclic heifers at day 8–10 of estrous cycle. Further 8 cyclic heifers were selected for present study on day 0 (estrus phase) and day 10 (diestrus phase) of estrous cycle. The estrum was confirmed by an expert and concerned veterinarian at the farm. The animal experiments were performed in September to December, with mean maximum temperature 27.4°C and minimum 15.2°C and were acceptable to the ethical standards of the National Dairy Research Institute, Karnal, India vide IAEC No. 23/09-21/11/2009.

Blood was collected from the jugular vein in heparinised vacutainers in the morning between 7–8 AM. Total leukocyte count (TLC) and differential leukocyte count (DLC) was done immediately after collection as described by Schalm *et al.* (1975). Plasma was separated by centrifugation at 2500rpm for 30 minutes and stored at –20°C in aliquots of 1 ml in microcentrifuge tubes till further investigations. The lymphocytes from peripheral blood were isolated by gradient centrifugation using Histopaque–1077 as described by Young *et al.* (2005) with minor modification like gradient: cell suspension ratio of 1: 3, centrifugation at 1,800 rpm for 40 min. Lymphocyte proliferation response was determined by culturing the lymphocytes *in-vitro* in culture medium RPMI 1640 supplemented with 10% foetal calf serum and antibiotics. Phytohemagglutinin @ 5µg/ml culture medium was used as a mitogen and incubation for 36 h was achieved at 37°C and 5% CO_2 in a humidified CO_2 incubator. The proliferative response of lymphocytes was estimated using the colorimetric MTT (tetrazolium) method described by Mosmann (1983). Proliferation index was calculated by dividing absorbance of cells with mitogen by absorbance of cells without mitogen.

Plasma nitric oxide was estimated as total nitrite (NOx) using modified Griess reaction (Miranda *et al.* (2001). Acetonitrile was used for deproteinization of plasma proteins (Ghasemi *et al*. 2007). Every sample was evaluated in duplicate for absorbance at 540nm wavelength in ELISA plate reader. The concentration was calculated from the standard curve using linear regression equation. Inter assay and intra assay difference was 3.4 and 4.62% respectively. Plasma IgG levels were estimated by using bovine IgG ELISA core kit. Plasma tumor necrosis factor α (TNFα) was evaluated by using bovine TNFα ELISA kit. Plasma xanthine oxidase was estimated by using xanthine oxidase assay kit. Plasma sex steroid hormone, estradiol 17α, progesterone and testosterone, levels were determined by RIA using ^3H labelled tracers. Antisera used in present study for estrogen (anti 17α estradiol antibody produced in rabbit) and testosterone (anti 5α- dihydrotestosterone developed in rabbit) were purchased commercially. Progesterone antiserum (AS P4, 24.12.93) was a gift from Dr B S Prakash, Formerly Head, Animal Physiology, NDRI, Karnal, India.

Plasma progesterone was estimated by a direct RIA developed by Kamboj and Prakash (1993). However, estrogen and testosterone was extracted with benzene and diethyl ether respectively. After complete evaporation of benzene and ether, steroids were solubilised in RIA buffer (0.05 M Tris-HCL, pH 8, containing 0.1M NaCl, 0.1% gelatin and 0.1% sodium azide) and quantified by using RIA technique with counting of γ-radiation in Beckman γ counter, USA routinely used in the laboratory.

Thiobarbituric acid reactive substance (TBARS), an index of oxidative stress, was evaluated by the method described by Asakawa and Matsushita (1980). The ferric reducing ability of plasma (FRAP), a measure of antioxidant power, was determined as per Benzie and Strain (1996).

The data generated in present study were subjected to analysis using Systat 12 software package. Analysis of variance of the data was performed using 2- way ANOVA with variables age, sex included in the model as fixed effects and Tukey's Honestly-significant difference test was employed. Student's t test was also employed while comparing 2 means of estrous and diestrous stages of reproductive cycle. Values are presented as mean±SE.

RESULTS AND DISCUSSION

Plasma levels of estradiol 17α and progesterone were detectable only in female cyclic buffaloes and not in other animals under study. Estradiol and progesterone levels were 10.41±0.61pg/ml and 0.28±0.05ng/ml at estrus and 7.16±0.70pg/ml and 2.21±0.13ng/ml at diestrus stage of estrous cycle, respectively, that differed significantly ($P<0.01$). The plasma levels of testosterone were detectable only in male buffaloes of all groups. The average plasma testosterone level in male buffaloes of group 1, 2 and 3 was 66.66±6.97, 216.66±19.24 and 883.13±43.94 pg/ml respectively that differed significantly ($P<0.001$) between the age groups. These findings were analogous with the findings of Shafie *et al.* (1982), Rao and Pandey (1982) in buffaloes. Present findings illustrated clear variability in sex steroids in post-pubertal buffaloes.

Lymphocyte proliferation index (PI) in different age groups of buffaloes of either sex recorded in present study is presented in Table 1. There was no significant difference in PI between the 2 sexes irrespective of age but significantly ($P<0.05$) lower PI (1.77±0.09) was registered in group 3 post-

Table 1. Various immune competence and oxidative stress markers in Murrah buffaloes of different sex and age

Parameters	Group 1 (male)	Group 1 (female)	Group 2 (male)	Group 2 (female)	Group 3 (male)	Group 3 (female)
Lymphocyte PI	$2.09^{a}\pm0.12$	$2.19^{a}\pm0.11$	$1.82^{ab\pm}0.10$	$2.21^{a\pm}0.13$	$1.92^{ab\pm}0.19$	$1.63^{b\pm}0.15$
IgG (mg/ml)	$21.38^{b}\pm0.37$	$20.77^{b\pm}0.38$	$21.15^{b\pm}0.38$	$21.15^{b\pm}0.37$	$22.68^{a\pm}0.37$	$22.72^{a\pm}0.37$
NO (µM/L)	$39.38^{a}\pm2.00$	$36.27^{b}\pm1.07$	$37.47^{a\pm}3.55$	$40.54^{a\pm}1.71$	$43.76^{a\pm}3.26$	$24.70^{b\pm}1.33$
TNFα (ng/ml)	$0.83^{a}\pm0.07$	$0.91^{a}\pm0.03$	$1.06^{a}\pm0.07$	$0.91^{a}\pm0.05$	$1.05^{a}\pm0.12$	$0.20^{b}\pm0.02$
XO (mU/ml)	$3.16^{cd\pm}0.17$	$2.91^{d}\pm0.31$	$4.21^{ab\pm}0.17$	$4.01^{ab\pm}0.12$	$3.62^{bc\pm}0.12$	$4.37^{a\pm}0.11$
TBARS (nM/ml)	$2.13^{cd}\pm0.09$	$2.62^{c}\pm0.27$	$1.20^{d\pm}0.14$	$1.91^{cd\pm}0.16$	$3.57^{b\pm}0.14$	$5.81^{a\pm}0.45$
FRAP (µM/L)	$312.08^{d\pm}6.02$	$359.97^{ab}\pm7.95$	$368.29^{ab}\pm5.76$	$380.65^{a\pm}7.39$	$326.41^{cd\pm}4.23$	$336.26^{bcd\pm}3.06$
TLC (1×10^{3})	$13.65^{b}\pm0.42$	$15.90^{a}\pm0.39$	$12.72^{b}\pm0.40$	$12.87^{b\pm}0.61$	$13.35^{b\pm}0.39$	$11.04^{c\pm}0.57$
Lymphocyte (%)	$76.33^{a}\pm0.70$	$71.55^{bc\pm}0.93$	$70.00^{c}\pm0.95$	$71.00^{bc}\pm1.87$	$73.61^{ab}\pm0.69$	$73.12^{abc}\pm1.55$
Neutrophil (%)	$21.19^{c}\pm0.46$	$24.44^{ab}\pm0.57$	$26.47^{a}\pm0.90$	$25.94^{ab\pm}1.59$	$23.30^{bc\pm}0.50$	$25.45^{ab\pm}1.39$

Values with different superscripts between columns within same row differ significantly (P<0.05).

Table 2. Various immune competence and oxidative stress markers at estrus and diestrus phases of reproductive cycle in Murrah buffaloes

Parameters	Estrus (d 0)	Diestrus (d 10)
Lymphocyte proliferation index	1.85±0.14	1.65±0.13
IgG (mg/ml)	$20.98^{b\pm}0.30$	$22.71^{a}\pm0.24$
Nitric oxide (µM/L)	$38.05^{a\pm}2.52$	$24.58^{b\pm}1.29$
TNFα (ng/ml)	$0.93^{a\pm}0.08$	$0.19^{b\pm}0.02$
Xanthine oxidase (mU/ml)	4.08±0.22	4.35±0.144
TBARS (nM/ml)	$3.97^{b}\pm0.52$	$5.82^{a\pm}0.40$
Total antioxidant power (µM/L)	332.51±1.84	336.79±10.76
Total leukocyte count (1×10^{3})	$13.00^{a}\pm0.94$	$11.15^{b\pm}0.57$
Lymphocyte (%)	76.31±2.04	73.62±1.41
Neutrophil (%)	23.00±1.96	25.62±1.32

Values with different superscripts between columns within same row differ significantly (P<0.05).

pubertal buffaloes when compared to groups 1 and 2 prepubertal buffaloes (2.14±0.09) irrespective of sex. There was significantly (P<0.01) negative correlation of PI with the age (r = –0.41). The cyclic females registered higher proliferation index at estrus than at diestrus, but statistically the difference was not significant between these 2 phases of estrous cycle (Table 2).

Lymphocyte proliferation response to mitogen stimulation has been regarded as a promising marker of cellular immune competence (Kristensson *et al.* 1994). Probably present study stands first in farm animals to investigate lymphocyte proliferation during different phases of life and physiology; hence little information is available with respect to PI in domestic animals of different age and sex. Joydip *et al*. (2011) reported no significant difference in lymphocyte PI among the milking buffaloes on the basis of milk production but reported significantly reduced PI in buffaloes subjected to heat stress as compared to control. Groups 1 and 2 prepubertal buffaloes irrespective of sex registered higher proliferation than group 3 post-pubertal buffaloes, which stands analogous with the reports of Hoskinson *et al.* (1990) in pigs. However, Young *et al*. (2005) reported that age of crossbred cattle at sampling did not influence PHA induced proliferation in lymphocytes. Further, buffaloes at estrus had higher proliferation index (1.85±0.13) than diestrus (1.65±0.13) which is in agreement with the observations made by Marco *et al.* (2009) in mice and Sugiura *et al.* (2004) in bitch. Pampori and Sujata (2012) have reported stimulatory effects of estrogen and suppressive effects of progesterone on buffalo lymphocyte proliferation. These reports supported present findings of increased lymphocyte proliferation at estrus stage of reproductive cycle that was estrogen dominated. A large amount of information supports the view that sex steroids regulated lymphocyte proliferation and it might be species specific. Further in present investigation, female buffaloes before puberty did not exhibit any significant change in lymphocyte proliferation but post-pubertal buffaloes exhibited variation in lymphocyte proliferation with evident changes in sex steroid milieu. Thus, there could be a possibility of sex steroids regulating cell-mediated immunity in buffaloes.

The plasma IgG levels recorded in present study for buffaloes of different age and sex are presented in Table 1. The IgG levels differed significantly (P<0.001) between the age groups irrespective of sex. Group 3 post-pubertal buffaloes registered significantly (P<0.01) higher (22.70±0.26mg/ml) plasma IgG levels than prepubertal group 1 (21.08±0.26mg/ml) and group 2 (21.15±0.26mg/ml) buffaloes. There was a positive correlation of plasma IgG levels with age (r = 0.6). Similarly among the cyclic buffaloes plasma IgG levels were significantly (P<0.01) higher at diestrus than estrus phase of reproductive cycle (Table 2).

The most abundant immunoglobulin is IgG, which provides majority of antibody based immunity (Pier *et al.* 2004). Quantification of peripheral blood IgG allows an insight into the general immune status of the animal. Very little information is available in cattle and buffaloes that could reveal age and sex related changes in IgG. Herr *et al.* (2011) reported decrease in IgG 8 weeks before parturition that recovered 4 weeks after parturition in cattle. Brenner *et al.*

(1995) reported a significant drop in average serum IgG levels, from 36.4 mg/ml during the luteal phase of the estrous cycle to 28.3 mg/ml around estrus in dairy cows and attributed the changes in IgG levels to the drop in progesterone. Present findings in principle confirm the observations made by Brenner *et al.* (1995) in cattle. Since the immunoglobulins are produced by B lymphocytes, relative change in the ratio of B and T lymphocytes during different stages of estrous cycle can be the reason for high Ig levels at diestrous than at estrus, and progesterone may be the candidate hormone in its regulation. Similarly IgG levels elevated with the age reported in present study probably reflect the maturation of humoral component of immune system.

Although NOx was significantly (P<0.01) higher in males (40.20±1.42µM/L) as compared to females (33.83±1.42µM/L) irrespective of age, yet there was no significant age related difference in plasma levels of NOx. Further, sex and group interaction indicated that NO concentration was significantly (P<0.001) low in groups 1 and 3 female buffaloes but no significant difference in group 2 male and female buffaloes. The group 3 post-pubertal males registered the highest (43.76±2.47 µM/L) NOx levels whereas females the lowest (24.70±2.47 µM/L) (Table 1). The plasma NOx levels were also found significantly (P<0.001) higher at estrus than diestrus phase of reproductive cycle (Table 2).

During the past 2 decades, nitric oxide (NO) has been recognized as one of the most versatile players in the immune system. It is involved in the pathogenesis and control of infectious diseases, tumors, autoimmune processes and chronic degenerative diseases. Bezooijen *et al.* (1998) reported that plasma NO was partly regulated by sex steroids in rats. In present study plasma nitric oxide was higher in males than females, with highest levels in post pubertal males. These results in buffaloes are in conformity to the findings of Pampori and Sujata (2012) who reported high levels of NO in culture supernatant of buffalo male lymphocytes than female lymphocytes. Present findings were comparable to the reported values by Zaher and Ahmad (2008), Khadrawy *et al.* (2008) in healthy buffalo cows. Appreciable levels of plasma NO as recorded in present study in buffaloes might be functioning as non-specific immune factor, providing resistance to infections for which buffaloes are recognized as disease resistant. The levels were also found to differ significantly (P<0.001) at different stages of estrous cycle with higher plasma NO at estrus (38.05±2.52 µM/L) when compared to diestrus (24.58±1.29 µM/L). These results are in total agreement with the findings of Dixit and Parvizi (2001), Aziz *et al.* (2008) in cattle and have attributed the variation in NO to the cyclic changes in estradiol and progesterone. Further, lowest levels of NO at diestrus as observed in present investigation may probably put buffaloes at a risk of infections at this stage of cycle.

Significant (P<0.001) difference in plasma TNFα was found between sexes and groups. Females registered significantly (P<0.001) lower values (0.67±0.04 ng/ml) for TNFα as compared to males (0.98±0.04 ng/ml) irrespective of age. Overall group effect was also significant (P<0.001), with groups 1 and 2 buffaloes registering higher 0.87±0.04 and 0.98±0.04ng/ml TNFα levels, respectively, than 0.62±0.04ng/ml in group 3 buffaloes irrespective of sex. Among the group 3 post-pubertal buffaloes, females registered the lowest TNFα (0.20ng/ml) which tended to differ significantly (P<0.001) from all other groups of buffaloes of either sex (Table 1). Significantly (P<0.001) high plasma TNFα was found at estrus than diestrus phase in cyclic buffaloes (Table 2).

The pro-inflammatory cytokine TNF-α is one of the most important proinflammatory mediator, which induces a number of different biological effects in various cells, initiating and regulating the immune responses (Bemelmans *et al*. 1996, Cavaillon *et al*. 2003). Several lines of evidence suggested that the outcome of some infections may be aligned with, and perhaps may be predicted by plasma levels of cytokines, particularly TNFα (Cavaillon *et al*. 2003). Significant (P<0.001) age and sex bias in TNFα levels was found during present investigation in buffaloes. The levels were high in young buffaloes of groups 1 and 2 as compared to group 3 post-pubertal buffaloes. Females had significantly (P<0.001) lower levels as compared to males irrespective of age, that might probably be responsible for augmented pathophysiological changes in males after sepsis as compared to females. Kahl and Elsasser (2006) reported that testosterone increases TNF-α and APP, which may be responsible for a differential presentation of disease symptoms between sexes or between steers and bulls. In present study the high levels of TNFα reported in group 3 males with highest levels of testosterone as compared to other age groups supported the findings of Kahl and Elsasser (2006). Jorg (1998) reported higher tendency of men to TNFα levels as compared to women after diagnosis of sepsis, Ying *et al.* (2008) reported low TNFα from PBMCs of females as compared to age matched males. Asai *et al*. (2001), Bouman *et al.* (2004) recorded more TNFα in males as compared to females in endotoxin stimulated monocytes. All these reports support the present findings in buffaloes. There is paucity of information on TNFα levels in healthy domestic animals. Horadagoda *et al.* (2002) recorded basal levels of plasma TNFα ranging from 0.4ng/ml to 0.7ng/ml in buffaloes that supported the present findings and it seem that buffaloes do maintain higher plasma TNFα levels necessary for maintenance of homeostasis. The TNFα levels in cyclic buffaloes were significantly (P<0.001) high at estrus than diestrous which in principle confirm association of sex steroids with immune mediation. In females puberty, pregnancy, menopause, and age have been shown to affect the immune response to a disease stress through the prevailing sex steroid milieu (Styrt and Sugarman 1991, Olsen and Kovacs 1996) and TNFα is the primary immune mediator

which is produced during stress. Progesterone has been entailed in transmitting immunosuppressive signals during pregnancy by decreasing production of inflammatory cytokines, particularly TNFα (Olsen and Kovacs 1996). Present findings along with the reported findings advocated the influence of sex steroids in the production of TNFα.

Plasma XO level recorded in buffaloes of different sex and age is presented in Table 1. Buffaloes of either sex exhibited no significant variation in enzyme concentration, however, significantly (P<0.001) low plasma XO was recorded in group 1 buffaloes (3.03±0.13) as compared to group 2 (4.11±0.13) and group 3 buffaloes (4.00±0.13mU/ml) irrespective of sex. Female group 1 buffaloes registered (P<0.01) the least plasma XO and female group 3 the highest (Table 1). Further, there was no significant difference in plasma XO at estrus and diestrus stages of estrous cycle in cyclic buffaloes (Table 2).

Xanthine oxidase (XO) is a complex molybdoflavo-enzyme, which uses xanthine, hypoxanthine, and reduced nicotinamide adenine nucleotide as electron donating substrates and catalyzes the last two steps in the formation of urate (Harrison 2002). It is also found as membrane-bound and free XO, which can generate weakly microbicidal superoxide and hydrogen peroxide (Bjorck and Claesson 1979). In present investigation, positive correlation was found between age and XO which is in agreement with the findings of Kuldip *et al.* (2009) in humans, thus suggesting increase in oxidative stress with age. Further, there was no significant difference in plasma XO at estrus (4.08±0.22mU/ml) and diestrous (4.35±0.14mU/ml) phase. The levels were comparatively higher in buffaloes than reported values for cattle (Chen *et al.* 1995). XO being a primary enzyme in purine metabolism, higher XO levels in buffaloes may have special role to play in nitrogen metabolism, which in ruminants has exceptional importance. XO converts nitrate into nitrite, and so may increase substantially the substrate source for NO generation (Li *et al.* 2003). Therefore, higher XO activity could be responsible for high NO levels in buffaloes because XO and nitrite levels were sufficient to generate NO at levels comparable with those from maximally activated nitric oxide synthases under normoxic or hypoxic conditions.

There was no significant difference found in total leukocyte count between sexes, with males and females registering $13.24\pm0.27\times10^3$ and $13.27\pm0.27\times10^3$ leukocytes per cubic mm of blood respectively. However, there was significant (P<0.001) difference between the age groups with group 1 buffaloes having highest total leukocyte count ($14.77\pm0.33\times10^3$) followed by group 2 ($12.79\pm0.33\times10^3$) and group 3 buffaloes ($12.20\pm0.33\times10^3$). Among the various groups of either sex, female group 1 buffaloes registered (P<0.001) the highest total leukocyte count and female group 3 the lowest (Table 1). Post-pubertal cyclic buffaloes exhibited significant (P<0.05) difference in TLC (Table 2) with higher counts at estrus than diestrus.

Differential count was made in respect to 2 major cells, lymphocytes and neutrophils. No significant effect of sex was found on differential leukocyte count. Males registered 73.31±0.69 and 23.65±0.58 whereas females 71.89±0.69 and 25.28±0.58% of lymphocytes and neutrophils, respectively, (Table 1). No significant difference in lymphocyte and neutrophil % was observed between estrus or diestrus stages of estrous cycle (Table 2).

Cellular components of blood, the WBCs are main props of both innate as well as adaptive immune system. While neutrophils are providing nonspecific but first line defence, the lymphocytes provide specific and long lasting immunity. The variation in counts of white blood cells is being used as an important and first hand diagnostic tool in animals as well as humans to detect any antigenic challenge. No significant difference was observed for TLC in male and female buffaloes in present study that is in agreement with the findings of Giltay *et al.* (2000), Bouman *et al.* (2004) in humans**.** However, age difference was significant with high TLC in young as compared to older which in principle agrees with the findings of Ferrer *et al.* (2000) and Beechler *et al.* (2009) in buffaloes, Pampori *et al.* (2010) in goats, Satue *et al.* (2009) in mares.

TLC was significantly high at estrus than diestrus stages of reproductive cycle which is comparable to the findings of Giltay *et al.* (2000). Further Giltay *et al.* (2000) reported increased mean number of leukocytes after estrogen plus anti-androgen administration to males, whereas no change occurred after testosterone administration to females. These findings uphold the present findings of high TLC value in estrus than diestrus stage of reproductive cycle, which is dominated by estrogen.

Significant (P<0.001) difference was observed in TBARS between sexes and groups. Female sex irrespective of age group registered higher values (3.45±0.14nM/ml) than male sex (2.30±0.14nM/ml). Post pubertal buffaloes of either sex registered significantly (P<0.001) higher (4.69±0.17nM/ml) values for TBARS as compared to group 2 (1.55±0.17nM/ml) and group 1 (2.37±0.17nM/ml) buffaloes. Group 2 male buffaloes registered (P<0.01) the lowest and group 3 females the highest values for TBARS (Table 1). Cyclic female buffaloes registered significantly (P<0.01) higher TBARS at diestrus than estrus (Table 2).

Plasma FRAP levels in various groups of buffaloes of either sex are presented in Table 1. The antioxidant power was significantly (P<0.001) higher in females (358.963±3.45μM/L) as compared to males (335.598±3.45μM/L) irrespective of age group. Group 2 buffaloes registered significantly (P<0.001) higher (374.475±4.23μM/L) antioxidant power as compared to group 1 (336.029±4.23μM/L) and group 3 (331.338±4.23μM/L) buffaloes irrespective of sex. Further there was no significant difference in antioxidant power between estrus and diestrus stages of reproductive cycle (Table 2).

Thiobarbituric acid reactive substances (TBARS) and ferric reducing ability of plasma (FRAP), a measure of lipid peroxidation and total antioxidant power, is now being extensively studied to assess the oxidative stress which provides a good reference of animal homeostasis. Evaluation of oxidative stress has contributed increasingly to our knowledge of the fundamental mechanisms involved in metabolic challenges, especially important in domestic animals in which many physiological processes like lactation, pregnancy impose physiological demands on animal homeostasis. Total antioxidant power is a single measure that aims to describe the dynamic equilibrium between pro-oxidants and antioxidants in the plasma compartment (Ghiselli *et al.* 2000). TBARS had a positive correlation with age as reported in present study in buffaloes, suggesting increased oxidative stress with increase in age. Cyclic females registered significantly (P<0.01) higher TBARS at diestrus (5.82±0.40nM/ml) than at estrus (3.97±0.52nM/ml) stage of reproductive cycle. There are reports of increased TBARS and decreased total antioxidant power in dairy cows in different stages of lactation (Castillo *et al.* 2006). Kuldip *et al.* (2009) reported significant increase in the level of lipid peroxidation while a decrease in the levels of antioxidant defence enzymes in plasma. All these findings supported the view of aging with increased oxidative stress. Present study in principle agrees with free radical theory of aging because lipid peroxidation was found positively correlated with age in this study. However, present study also supported the view that body tries to maintain homeostasis at all levels, and as the lipid peroxidation increased with age, there was also a positive change in total antioxidant power that probably helps in maintaining homeostasis. The use of TBARS and FRAP values, measures of oxidant-antioxidant balance, could provide complementary information about the homeostasis of the animal than conventional metabolic parameters alone.

Variability in the immune parameters like lymphocyte proliferation, immunoglobulins, TNFα, nitric oxide, xanthine oxidase or leukocytes between the age groups, sexes and phases of reproductive cycle accompanied by variations in sex steroids, strongly suggest the role of gonadal steroids in the immune-modulation.

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