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ORIGINAL PAPER

Feed supplementation prevents post-conception decline in milk progesterone concentrations associated with production stress in dairy buffaloes (*Bubalus bubalis*)

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Abstract The onset of pregnancy may be associated with hormonal changes and decline in milk yield of buffaloes. To investigate this, forty lactating buffaloes from 1st to 23rd weeks post-conception were selected. The animals were assigned to three treatments: PRT, PRS, NPRT and three milk yielding groups HMY, 66 to 75 l/week, n=12; MMY, 56 to 65 l/week, n=16; LMY, 46 to 55 l/week, n=12). Milk samples were collected on alternate weeks and analyzed with ultrasonic milk analyzer. EIA was used for milk progesterone concentrations. Group means were compared and correlation analysis was conducted. Progesterone concentrations increased in almost similar pattern with the advancing weeks post-conception. The high and low yielder showed greater progesterone concentrations in the supplemented than the animals on traditional ration ($P<0.001$). Progesterone concentrations correlated positively with fat (%), negatively with milk yield, protein (%) and lactose (%). Decline in milk yield became drastic when progesterone concentrations rose above 6.44 ng/ml. The pregnant animals on traditional ration exhibited a sharper decline in milk yield with the increasing progesterone concentrations as compared to pregnant animals with supplemented ration. It is concluded that concentrates supplementation induced a raise in progesterone levels. Progesterone concentrations and milk yield showed an inverse relationship.

Keywords Progesterone · Pregnancy · Milk yield · Feed supplementation · Dairy buffaloes

Abbreviations

PRT	Pregnant-ration traditional
PRS	Pregnant-ration supplemented
NPRT	Non-pregnant- ration traditional
HMY	High milk yielder
MMY	Moderate milk yielder
LMY	low milk yielder
EIA	Enzyme immunoassay
MPL	Milk progesterone level

Introduction

In buffaloes, progesterone profiles have been studied in post-partum periods. Following the first postpartum ovulation, plasma progesterone levels were found to increase and remain above 0.7 ng/ml for about 10 days; then, they declined to below 0.25 ng/ml at the next estrus. Milk progesterone concentrations

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were successfully used in estrus detection (Qureshi et al. 1989) and in the assessment of ovarian status (Qureshi et al. 1992) in Nili-Ravi buffaloes. Usmani et al.(1990) found that 86% of Nili-Ravi buffaloes showed at least 1 short luteal phase (8 to 13 days) before the first estrus. Demise of the corpus luteum after calving expressed by progesterone concentration on Day 3 postpartum was not different in milked and suckled buffaloes (Arya and Madan 2001). Progesterone assays has been used in buffaloes for detecting ovulations (Kaul, and Prakash 1994), diagnosing and treatment of reproductive disorders (Kaul et al. 1993) and pregnancy detection in buffaloes (Gupta and Prakash 1990). Qureshi et al. (2000) reported that low postpartum reproductive performance in dairy buffaloes during low breeding season (LBS) was primarily due to inadequate functioning of the corpus luteum in secreting optimum concentrations of progesterone than the normal breeding season (NBS). LBS in the study area occurs during the spring and summer months which is associated with availability of leguminous fodders. Crude protein intake (CPI) was higher during LBS ($P<0.01$) and was positively correlated with serum urea levels ($r=0.22$, $P<0.01$). Degradable protein intake (DPI) showed the same seasonal pattern. ($P<0.01$; summer>spring>autumn/winter). CPI excess to requirements was lower in animals which expressed oestrus than those which remained anoestrus ($P<0.05$).

The higher incidence of silent estrus during low breeding season indicated improved management requirements for the detection of estrus. El-Wishy (2007) reviewed endocrinological changes and uterine involution in postpartum buffaloes and reported relationship between the patterns of estrogens and progesterone in the last stages of gestation and during calving in relation to placental delivery or retention, uterine torsion and degree of cervical dilation as well as response to exogenous glucocorticoids for induction of parturition and recommended further studies. A biphasic rising pattern of progesterone concentrations postpartum was noted in dairy buffaloes (Qureshi et al. 2008) with a slight increase during the first month postpartum followed by a rapid increase during the second month; a plateau up to fourth month and than again a rapid increase onwards.

In dairy cows, relationship of milk progesterone levels with milk yield has been reported to be negative during both the first and third luteal phases postpartum in second parity cows (Reksen et al. 2002). Negative energy balance was associated with reduced values for milk progesterone during the third luteal phase in second parity cows. An association was demonstrated between likelihood of conception and the energy coverage in Norwegian cattle; possibly mediated through progesterone deficiency. Decline in milk yield after the peak is associated primarily with decline in cell numbers due to apoptosis (Knight and Wilde 1993), decline in milk yield during pregnancy is associated primarily with decline in milk synthesis and rate of secretion accompanying the increase in progesterone (Forsyth 1999).

Information on relationship of progesterone levels with milk yield in dairy buffaloes is not available. The drastic decline in milk yield with the onset of pregnancy in this animal has been observed by farmers under traditional system with the primary aim to produce milk for utilization by the urban and rural populations. This type of farming in the private sector of Pakistan has been described (Qureshi 1995) where buffaloes are kept under a low input dairy farming. There is no practice of feeding animals according to requirements; exposing them to nutritional deficiency with the onset of pregnancy. It leads to a decline in milk yield, which compels the farmers to keep the animals un-bred. Our group (Qureshi et al. 2007) concluded that the onset of pregnancy in dairy buffaloes results in drastic decline in yield at an early stage and the high yielders are more sensitive.

As the onset of pregnancy is associated with an increase in progesterone concentrations and there is also a drastic decline in milk yield; this study was conducted to investigate the relationship among the feeding, milk yield, pregnancy and progesterone concentration.

Materials and methods

Animal selection and management

This study was conducted at a medium-sized private buffalo farm, during the July 2005 to December 2005. The farm was located in the Central Valley of the NWF Province of Pakistan lying at 31 to 37°N and 65 to 74°E. The animals were selected for the experiment within 60 to 90 days after parturition. All experimental animals were stall-fed and provided green fodder *ad lib*. Water shower was provided to all animals during hot season twice a day at the farm. Drinking water was provided three times daily. Animal sheds were washed twice daily at morning and evening. Twice a day milking was practiced at about 4 AM and 4 PM. Vaccines against hemorrhagic septicemia and Foot and Mouth Disease were administered to all experimental animals, as per prevailing practice. Anthelmintic drench of Levamisole Hydrochloride plus Oxychlosanide according to manufacturer instructions was provided at the start of the experiment.

Feeding regime and milk sampling

Basal ration comprised green fodder *ad lib* during June through October including maize, sadabahar (sorghum x sudan grass) and sorghum. During November to May, Egyptian clovers, oats, brassica and wheat straw were offered.

i) Ration Traditional (RT): In addition to the green fodder, all animals were provided a commercial concentrate; having 18% crude protein (CP) and 72% total digestible nutrients (TDN) at the rate of 1.5 kg per animal irrespective of its lactation stage, milk yield level and pregnancy stage, as per routine practice under the conventional farming system in the region. This constituted ration for all the non-pregnant and one group of pregnant experimental animals. Pregnancy diagnosis of the experimental animals was made on 21 days post-breeding through milk progesterone EIA.
ii) Ration supplemented (RS): In addition to the basal ration, the same commercial concentrate was provided at the rate of 1 kg per 2 liter of milk as recommended by Ranjhan (1994) for lactating buffaloes under tropical conditions. Supplemented ration was provided to one group of pregnant animals.
iii) Milk sampling: Milk samples, 20 ml each were collected from evening milk of all experimental animals on alternate weeks just after insemination, till the cessation of lactation.
iv) Milk composition: Milk samples (10 ml each) collected from the experimental animals was utilized for composition determination. Milk contents were determined through ultrasonic milk analyzer (model Ekomilk Total Ultrasonic Milk Analyser, Bullteh 2000, Stara Zagora, Bulgharia), using manufacturer's instruction.
v) Milk progesterone assay: Milk samples (10 ml each) collected from experimental buffaloes was utilized for milk progesterone assay. Sodium azide 200 μl in 0.1 percent concentration was added to each milk sample as preservative. Samples with preservative were stored at –20 °C till its use for progesterone assay. Milk progesterone was determined through ELISA as described by Qureshi et al.(1992). The intra- and inter-assay coefficients of variation were 3.54% and 9.21%, respectively. The sensitivity (detection limit) of the assay was 0.09 ng/ml.

Experimental design and statistical analysis

The experimental animals were subjected to various treatments as given in Table 1. The data was analyzed as follows:

Post conception milk progesterone pattern:

Progesterone concentrations at various weeks post-partum as affected by groups, treatments, post-conception weeks, and their interactions were worked out. The data were split in two sets for combined analysis, set one consisted data from weeks 2 to 8 and set two comprised data from weeks 10 to 22. Some sum of squares (SS) for interaction in the combine ANOVA for even weeks were negative, so regression

Table 1 Number of experimental animals in various production and feeding groups*

Feeding group	Production group			Total
	HMY	MMY	LMY	
PRS	3	5	3	11
PRT	3	6	3	12
NPRT	6	5	6	17
Total	12	16	12	40

HMY: High yielders, 66 to 75 L/wk (n=12×23 weeks); MMY: Moderate yielders, 56 to 65 L/wk (n=16×23 weeks); LMY: Low yielders, 46 to 55 L/wk (n=12×23 weeks); PRT: Pregnant-Ration traditional, n=12×23 weeks; PRS: Pregnant-Ration supplemented, n=11×23 weeks; NPRT: Non Pregnant-Ration traditional, n=17×23 weeks approach was used to calculate SS for the sources of variation. Full model and reduced models were fitted to the data for progesterone concentrations during weeks 2 to 8 and weeks 10 to 22, separately to calculate SS for the different sources of variation in ANOVA using the following model.

$$Y = \mu + G + T + GT + W + GW + TW + GTW \quad (1)$$

Where Y is MPL; μ is overall mean, G is milk production class, T is treatment, W is post-conception week and GT, GW and GTW are the respective interactions.

Relationship of progesterone concentrations with various parameters:

Correlation analysis was conducted for determination of correlation coefficient of progesterone concentrations with milk yield fat%, SNF%, protein%, lactose% and ash% using the following model:

$$rx, y = cov(X, Y)/\sigma x \sigma y \quad (2)$$

Where $r\, x_{,\ Y}$ is the correlation coefficient, X and Y are the parameters, σ_x and σ_Y are variance of the respective parameter.

Progesterone interacts with milk yield:

The decline in milk yield also showed a critical point beyond which it became comparatively rapid. So the trends of milk yield as affected by progesterone concentration were analyzed using the following regression model based on joining point of the two phases:

$$Y = a + bP + cP' \quad (3)$$

Where Y is decline in milk yield: a, b and c are constant: P is progesterone; P' is 1 for P ≤ JP and P-JP for P> JP. JP is joining point.

Results

Post conception milk progesterone pattern

Milk progesterone concentrations did not vary among the production groups during the initial 8 weeks but later on the difference became significant (Table 2). It was probably due to the overall lower quantity of progesterone concentrations initially which later on increased, making the dilution factor and the difference in progesterone concentrations larger. Treatment had a significant effect on progesterone concentrations during both the initial and later phases. During the initial phase the non-pregnant animals showed lower progesterone levels than the pregnant ones while during the later phase the concentrates supplementation supported progesterone concentrations through better metabolic support. Post-conception week showed a significant variation in progesterone concentrations due to advancement in embryonic development and implantation supported by increasing size of luteal tissues and other sources.

Interaction of production groups was significant with treatments during the 2–8 weeks and with weeks during 10–23 weeks post-conception. Treatment x

Table 2 Combined ANOVA for milk progesterone levels (ng/ml) during various weeks post-conception

SOV	Phase I (week 2–8)			Phase II (week 10–22)		
	DF	MS	P	DF	MS	P
Production group (G)	2	0.445	0.100	2	9.0917	0.000
Treatments (T)	2	117.230	0.000	1	43.073	0.000
G x T	4	1.138	0.000	2	0.167	0.766
Error-I	31	0.179		17	0.619	–
Weeks (W)	3	8.569	0.000	6	160.977	0.000
G × W	6	0.306	0.145	12	1.096	0.002
T × W	6	8.162	0.000	6	0.221	0.762
G × T × W	12	1.951	0.000	12	0.247	0.817
Error-II	93	0.187	–	102	0.396	–
Total	159	–	–	160	–	–

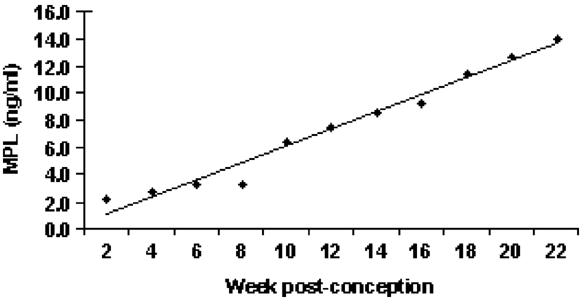

Fig. 1 Milk progesterone levels (MPL) during post-conception period in dairy buffaloes (y = 1.1199x + 0.9465; R2=0.9847)

week interaction was significant only during 2–8 weeks. Interaction among production groups and weeks was significant and with the advancement of pregnancy the progesterone concentrations increased in all groups. Treatment interaction with the production groups and post-conception week was significant during the initial phase. However, later on the treatment confounded the effect of production groups, post-conception week and both of them jointly, providing support to progesterone concentrations in different ways.

Figure 1 shows that the progesterone concentrations rise slowly during the initial phase from week 2 to week 8 post-conception. Later on the rate of enhancement increases rapidly which may be due to enhanced production of progesterone to support the developing and implanting embryo from corpus lutum as well as placenta.

Figure 2 shows that progesterone concentrations increases in a similar pattern with the advancing weeks post-conception in all the three production groups; however the progesterone levels were slightly but constantly higher in LMY followed by MMY and HMY. It may be due to the decreasing concentration of progesterone with the increasing volume of milk.

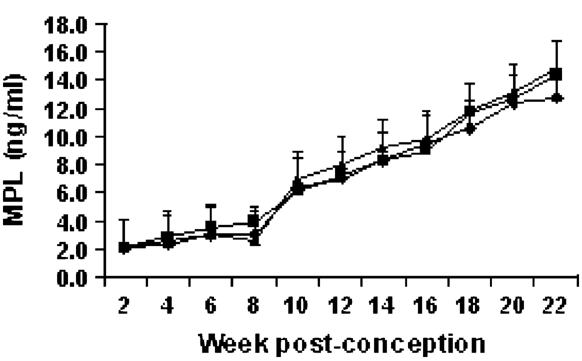

Fig. 2 Milk progesterone levels (MPL) in high (♦), moderate (■) and low (▲) yielding buffaloes (2–8 weeks: P>0.01; 10–23 weeks: P<0.01)

Effects of feeding status

Figures 3A–C report the changes in progesterone concentrations in the three treatments PRS, PRT and NPRT animals belonging to three production groups. The non-pregnant animals showed lower progesterone

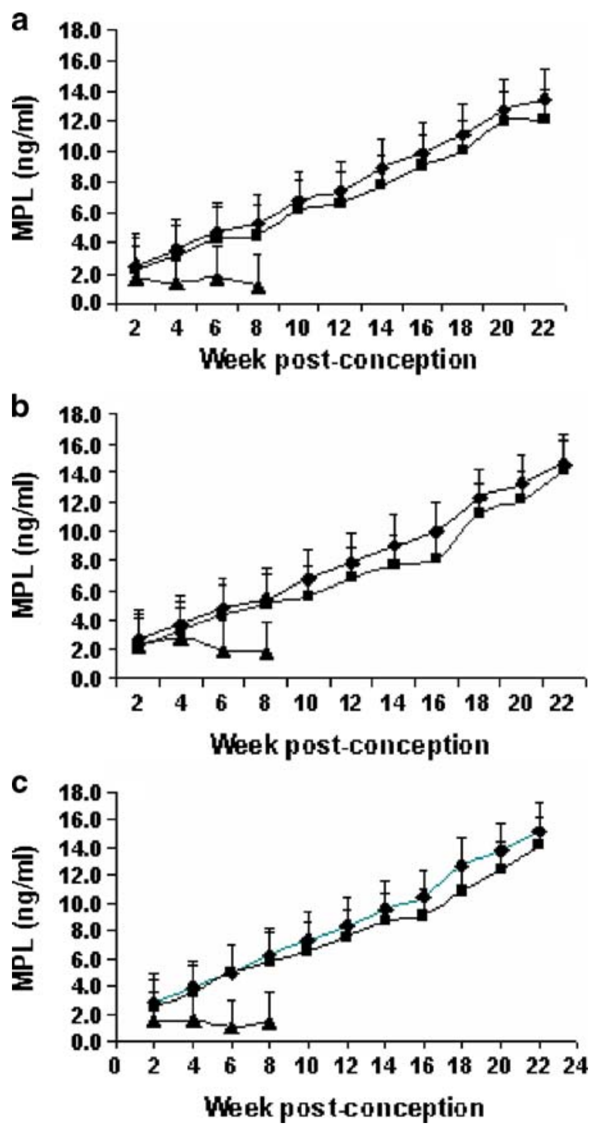

Fig. 3 A to 3-C Milk progesterone levels (MPL) in PRS (♦), PRT (■) and NPRT ▲) high (3-**A**), moderate (3-**B**) and low (3-**C**) yielding buffaloes

concentrations which was sampled only up to 8 week post-conception. Pregnant buffaloes offered with PRS in HMY and LMY groups showed greater progesterone concentrations than the animals provided with traditional ration ($P<0.001$). These findings suggest that concentrates supplementation raises progesterone levels in high and low yielders.

Relationship between progesterone, milk yield and composition

Correlation of progesterone concentrations with various milk parameters are given in Table 3. Progesterone concentrations correlated positively with fat (%) and negatively with milk yield, protein (%) and lactose (%) constants. An advancing progesterone concentration means an advancing gestation where the conceptus development regulates nutritional partitioning among the fetus and the dam. So more fat while little protein and lactose are secreted with the rising progesterone concentrations. The negative correlation of progesterone concentrations with milk yield is probably due to the dilution effect of progesterone with the increasing milk volume.

Figure 4 reports the association of milk yield with the increasing progesterone levels. There was a mild decrease in milk yield with the increasing progesterone levels up to 6.44 ng/ml (joining point of the two phases) but further increase in the progesterone concentrations shows drastic decrease in milk yield.

Figure 5 shows that the animals with traditional ration exhibited a sharp decline in milk yield with the increasing progesterone levels, in an un-interrupted trend. However, the concentrate supplemented animals showed the decline in two phases. With the increasing progesterone concentrations from 2.0 to 5.84 ng/ml the decrease in milk yield was zero. Further increase in progesterone concentrations

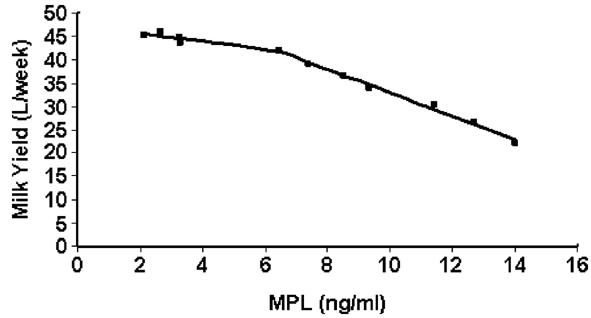

Fig. 4 Changes in milk yield with the increasing milk progesterone levels in dairy buffaloes. (Y=47.41937 - 0.88148 P - 1.59907 P'; For MPL < 6.44: P'=0; $R1^2$=0.761, Y=-0.8237X + 46.763; and for MPL > 6.44: P'=P - 6.44; R2=0.9875; Y=-3.3822 X + 43.328; Joining point of the two lines is 6.44, the critical point defining the drastic decline in milk yield)

resulted in a decreasing milk yield with a comparatively slower rate than the buffaloes on traditional ration.

Figure 6 show that all the high, moderate and low milk yielding buffaloes exhibit a decline in milk yield with the increasing progesterone levels beyond 6 ng/ml. However, this decline was faster in higher yielders followed by moderate and low yielders.

Discussion

Post conception milk progesterone pattern

The present study indicated a post-conception increase in MPL in two phases in dairy buffaloes; an early sluggish increasing phase of 8 weeks followed by a rapid increasing phase. Similarly, in dairy cows early in pregnancy, the progesterone concentration curve appears to be biphasic over time: increasing during 3 to 12 days after mating, leveling off until

Table 3 Relationship (correlation coefficients “r”) of milk progesterone levels (MPL) with various Parameters in dairy buffaloes

Parameters	Fat%	SNF%	Protein%	Lactose%	Ash%	MPL
Milk Yield	-0.5284***	0.0211	0.3184***	0.1683**	0.0418	-0.6083***
Fat%	–	-0.0688	-0.4744***	-0.3565***	-0.0927	0.9080***
SNF%	–	–	-0.0087	0.0759	-0.0031	-0.0691
Protein%	–	–	–	-0.1383*	0.0161	-0.4995***
Lactose%	–	–	–	–	-0.0218	-0.3611***
Ash%	–	–	–	–	–	-0.0623

* Significant ($P<0.05$) ** Significant ($P<0.01$) *** Significant ($P<0.001$)

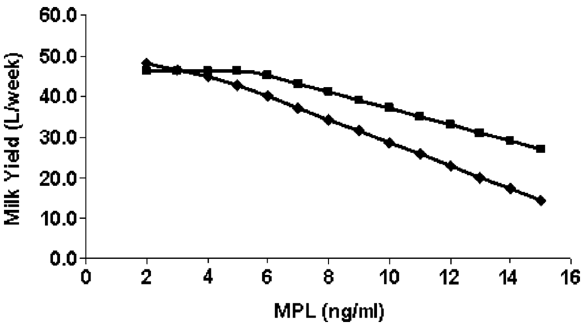

Fig. 5 Changes in milk progesterone levels (MPL) with the increasing milk yield in buffaloes; PRS (■) and PRT(♦) buffaloes (■ Y1=57.1677 - 2.0099P'; R-square=0.9901; ♦ Y2=51.4432 - 1.5946P - 1.2549P''; R-square = 0.9910)

about 30 days, and increasing further to 39 days (Henricks et al. 1972). There is a positive association between blood progesterone concentrations and pregnancy (Lamming and Darwash 1998). Sousa et al. (1999) investigated blood progesterone levels in pregnant goats and found that it differed significantly between the 1st and 3rd week of gestation. Later changes within the two- week intervals were not that distinct, although in goats of the Canadian breed the blood progesterone level was also increasing between the 7th and 11th week of gestation.

In agreement to the present study, our previous investigations (Qureshi and Ahmad 2008) found a slight increase in MPL during the first month postpartum followed by a rapid increase during the second month; a plateau up to 4th month and than again a rapid increase onwards. In dairy buffaloes Singh and Puthiyandy (1980) reported that progesterone concentration in the milk of pregnant animals (24.83±3.85 ng/ml) was significantly higher than that of non-pregnant animals (2.89±1.21 ng/ml) on Day 20 and the difference between the two increased with time after insemination. Progesterone concentration was found to be positively correlated with corpus luteum size (Qureshi et al. 1992) and the corpus luteum was not efficiently working to produce sufficient progesterone for reproductive cyclicity during summer (Qureshi et al. 2002). The lower progesterone levels reduced the response of buffaloes to hormonal therapy of infertility (Singh et al. 2006). The overall LH response to GnRH was much lower in luteal-phase buffaloes with elevated progesterone concentration than in non-cycling buffaloes with smooth ovaries and basal progesterone concentrations, irrespective of their suckling status.

Feed supplementation and MPL

The present results indicate that concentrates supplementation raises progesterone levels in high and low yielders. In the HMY animals there may be production stress which may lower the MPL and this lowering trend is prevented by the concentration supplementation. In LMY buffaloes there seemed to be the stress of overfeeding of degradable protein (Qureshi et al. 2002) as they were fed at the same scale under conventional feeding regime, at par with the high and moderate yielders; and they could not utilize the excess intake of protein. In the supplemented buffaloes the animals were fed according to the feed requirements, there was no question of overfeeding which resulted in enhanced MPL.

In line with our findings, Lucy (2001) has reported that under-nutrition or negative energy balance may compromise pregnancy through its effect on the corpus lutem. Higher milk yielding cows had lower progesterone concentrations, associated with infertility. In another study, cyclic underfed cattle had progressively smaller and less estrogenic dominant follicles before they succumb to anestrous (Bossis et al. 1999). The smaller dominant follicles gave rise to smaller corpora lutea. Steroidogenic capacity of luteal cells is also dependent on hormones such as somatotropin, insulin and IGF-I that are controlled by the nutrition of the cow (Lucy 2000). Association of

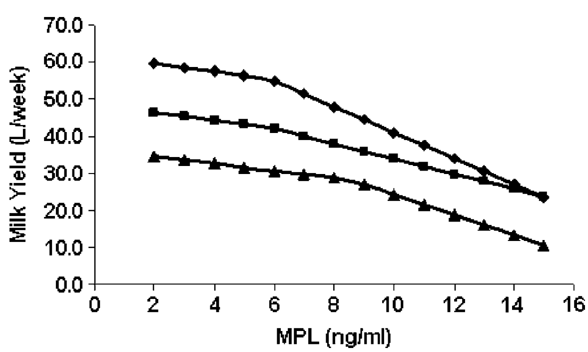

Fig. 6 Milk progesterone levels (MPL) change with milk yield in high (♦), moderate (■) and low (▲) yielding buffaloes. (♦ Y1=61.8977 - 1.0976P - 2.3694 P''; $R1^2$=0.9826; ■ Y2= 48.5818 - 1.0665P - 0.9689 P''; $R2^2$=0.9911; ▲ Y3=36.6094 - 0.9923 P - 1.7256P''; $R3^2$=0.9949)

progesterone levels with milk yield acting via nutritional status has been suggested (Lucy 2001). Under nutrition or negative energy balance may compromise pregnancy through its effects on the corpus luteum. High-producing dairy cows had lower blood concentrations of progesterone and the lower blood progesterone concentration were attributed to infertility.

Mechanism of action of nutritional stress associated with body weight loss and lower circulating progesterone concentrations has been attributed to selection for increased milk yield (Lucy and Crooker 2001). It has been hypothesized that growth and development of follicles during periods of negative energy balance lead to impaired development of the CL and a reduction in progesterone secretion (Butler 2000). Cows that produce more milk have smaller CL at the peak of lactation (Lucy 2000) and CL size has been correlated positively with circulating progesterone concentration. However, circulating progesterone concentration is determined by rates of secretion and clearance. Clearance rates of progesterone increase with feed intake due, in part, to an increase in hepatic metabolism (Sangsritavong et al. 2002). Therefore, at least two factors (CL size and feed intake) appear to be responsible for the reduction in circulating progesterone in cows that produce more milk. The addition of fat to the rations of dairy cows resulted in an increase in the levels of progesterone in blood (Lucy et al. 1993).

Similarly summer stress has been shown to lower MPL (Qureshi et al. 2000) in dairy buffaloes. MPL was found highest in spring (3.00±0.12 ng/ml) followed by winter (1.77±0.32), autumn (0.84±0.72 ng/ml), summer (0.25±0.04) ($P<0.01$). Summer contributed to the low breeding season (LBS) when the MPL did not reach the optimum levels until day 84 postpartum, indicating that the corpus luteum did not function efficiently to maintain high progesterone levels required for reproductive cyclicity. Interestingly, the incidence of silent ovulation in the buffaloes was higher in LBS than in NBS (70.6 vs 29.4%, respectively). Qureshi et al. (1999) found that MPL showed a pattern opposite to atmospheric temperature. Similarly, in a study on 17 complete postpartum periods in Murrah buffaloes in Sri Lanka, plasma progesterone concentrations remained basal (< 0.25 ng/ml) for a period ranging from 92–210 days (Perera *et al*. 1984). In Swamp buffaloes (Perera 1982), it was found that postpartum anestrus was due to a failure in the resumption of ovarian cyclicity in the suckled buffaloes. Kaur and Arora (1984) concluded that malnutrition coupled with high environmental temperature stress was responsible for long anestrous periods in buffaloes.

Progesterone interacts with milk yield and composition

Our study defined the critical level of 6.4 ng/ml of MPL associated with drastic decline in milk yield while the two parameters also showed a constant inverse relationship in buffaloes. Previous studies on cows have reports of the effect of progesterone during pregnancy on decrease in milk yield, by about 150 days of gestation (Coulon et al. 1995). Grossman and Koops (2003) concluded that yield rises rapidly to a peak as secretion rate increases, maintains a level for a period of time, and then decreases until the end of lactation as cell number decreases due to apoptosis and as secretion rate decreases due to pregnancy. Decline in milk yield after the peak is associated primarily with decline in cell numbers due to apoptosis (Knight and Wilde 1993), decline in milk yield during pregnancy is associated primarily with decline in milk synthesis and rate of secretion accompanying the increase in progesterone (Forsyth 1999).

Milk composition begins to change around parturition, the timing depending on the species, and gradually approaches the composition of true milk. Much of this change in composition can be attributed to closure TJ (tight junctions) Neville 1995). It was found that the mammary TJ, particularly in the alveoli, are leaky during pregnancy and close around parturition to form a tight barrier that prevents paracellular movement of molecules across the mammary epithelium. The hormonal regulation of tight junction closure *per se* has not been studied. However, lactogenesis itself is thought to be triggered by progesterone withdrawal and depend on the presence of glucocorticoids and prolactin (Neville et al. 2001).

The present study indicated a post-conception increase in progesterone concentrations in two phases in dairy buffaloes; an early sluggish increasing phase of 8 weeks followed by a rapid increasing phase. Concentrates supplementation raised progesterone levels probably through reducing production stress. Milk progesterone concentrations and milk yield showed a constant inverse relationship in buffaloes.

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