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CONTENTS

	Page
Case Report	
Colonic faecolith and its surgical management in a she buffalo <i>V.S. Dabas, D.N. Suthar, S.K. Jhala, C.F. Chaudhari, N.F. Chaudhari and D.C. Patel.....</i>	157
Dystocia due to emphysematous fetus and fetal maldisposition in a buffalo <i>Pravesh Kumar, Amit Sharma, Madhumeet Singh, Pankaj Sood and Jitender Agarwal.....</i>	160
Short Communication	
Efficacy of deltamethrin against lice infestation in buffaloes calves <i>K. Arunachalam, M. Raman, T.J. Harikrishnan and T. Anna.....</i>	162
Original Article	
Follicular dynamics and uterine status after synchronization of ovulation in early post-parturient Egyptian buffaloes <i>M.M. Kandiel, B.A. Gad, G.A. Sosa and A.I. El-Azab.....</i>	165
Effect of TCM-199 and synthetic oviductal fluid (SOF) medium and cysteamine supplementation to in vitro maturation media on maturation, cleavage rate and subsequent embryonic development of buffalo oocytes <i>Ch. Srinivasa Prasad, A. Palanisamy, V.S. Gomathy, S. Satheshkumar, A. Thangavel and G. Dhinakar Raj.....</i>	182
Evaluation of the superovulatory effect of pregnant mare's serum gonadotropin (PMSG) and follicle stimulating hormone (FSH) in the non-breeding season of river buffaloes of the Miankaleh peninsula, northern Iran <i>Behrang Ekrami, Hamid Ghasemzadeh-Nava, Parviz Tajik and Amin Tamadon.....</i>	189

CONTENTS

Page

Original Article

Molecular characterization of the leptin gene in riverine buffaloes

Sanjoy Datta, Archana Verma, Paresh Chatterjee and Aruna Pal (Chatterjee).....196

Endocervical cytological studies in endometritis affected

Murrah graded buffloes (*Bubalus bubalis*)

K. Ramesh Babu, K. Mouli Krishna and K. Padmaja.....212

Field level study on the buffalo bullock: An excellent draught animal

Aruna Pal and P.N. Chatterjee.....218

Effect of feeding differently processed sweet sorghum bagasse based complete rations
on feeding behaviour, milk production and cost economics in graded Murrah buffaloes

Ch. Venkata Sessaiah, S. Jagadeeswara Rao, Y. Ramana Reddy,

M. Mahendar and M. Kishan Kumar.....231

COLONIC FAECOLITH AND ITS SURGICAL MANAGEMENT IN A SHE BUFFALO

V.S. Dabas¹, D.N. Suthar, S.K. Jhala, C.F. Chaudhari, N.F. Chaudhari and D.C. Patel

ABSTRACT

This communication reports a case of surgical management of colonic faecolith in a she buffalo.

Keywords: Mehsana buffalo, *Bubalus bubalis*, faecolith, colon

INTRODUCTION

Intestinal obstruction in ruminants is a sporadic and relatively infrequent condition. However, it is more common in cattle and sheep when compared with buffaloes and usually occurs in the jejunum and ileum and rarely in the colon (Singh *et al.*, 2003). Faecoliths instigated intestinal obstructions are usually encountered in bovines, wherein the undigested food material obstructs the tract and creates difficulties while completing the mechanism of digestion. The present report describes a successful surgical management of a rarely occurring colonic faecolith in a Mehsana she buffalo.

CASE HISTORY AND CLINICAL OBSERVATION

A three-year-old Mehsana she buffalo was presented with the history of anorexia and cessation of defecation for one week. Further, the animal was treated thrice by field veterinarians without any improvement in the condition. Clinically, the animal was dull, depressed, dehydrated and showed nasal discharge along with bilateral abdominal distention. Per-rectal examination revealed only mucous flakes and gas filled distended intestinal loops. A hard circular mass could be palpated at pelvic brim, and this was confirmed to be the intestinal obstruction after ultrasonographic examination (Figure 1).

SURGICAL INTERVENTION

The buffalo was restrained in the trevis and the right para-lumbar fossa was prepared for aseptic surgery in standing position. The abdominal cavity was opened through a 15cm long incision under local infiltration with 2% lignocaine hydrochloride. On exploration, a hard colonic mass was palpated and with great difficulties it was brought to the operative site. Entrotomy (Figure 2) was performed to remove the mass (faecolith) following principles of intestinal surgery. The entrotomy wound was

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closed by single layer of cushioning sutures using chromic catgut #1/0 with swagged on needle. The abdominal wound was closed in routine manner. It was interesting to note that the animal passed large quantity of the faeces even before completion of the skin sutures (Figure 3). Post-operatively, inj. 0.9 % NSS and inj. 5% DNS IV 2000 ml each along with antibiotic¹, analgesic² and antihistaminic³ were administered for five days in prescribed doses. Daily wound dressing using povidine iodine led to uneventful recovery and skin sutures were removed on the 12th postoperative day.

DISCUSSION

Any mechanical or functional interference with the progression of the intestinal contents cause obstruction (Singh *et al.*, 2003). Although volvulus, intussusception, adhesions or herniation are the common causes of intestinal obstruction; the faecoliths and foreign bodies have also been found responsible for this condition (Hofmeyer, 1982 and Kamble *et al.*, 2008). Faecoliths are predominately observed during summer months due to minimum availability of drinking water and simultaneous dry fodder feeding. However, the buffalo in the present study was admitted to the hospital in the month of January. Earlier, the diagnosis of intestinal obstruction was mostly based on the history, clinical signs and clinicopathologic examinations but imaging tools like ultrasonography with its

1 Trimethoprim and Sulphamethoxazole: Inj. Oripriam-V-Vet, Zydus Animal Health Ltd, Ahmedabad.

2 Meloxicam: Inj. Melonex, Intas pharmaceuticals Ltd., Ahmedabad.

3 Pheneramine maleate: Inj. Avil, Intervet Pvt Ltd, Wagholi

limited use in large animal abdominal imaging has also been reported (Braun, 2003) and served excellently in the present study. Successful surgical management of faecolith cases in cattle has been reported by Abutarbush *et al.* (1983) and Kamble *et al.* (2010). In the present case too; the recovery was uneventful but the vital part was the single layer intestinal suturing which would have been beneficiary over the diaphragm formation which in turn prevents the narrowing of the intestinal lumen.

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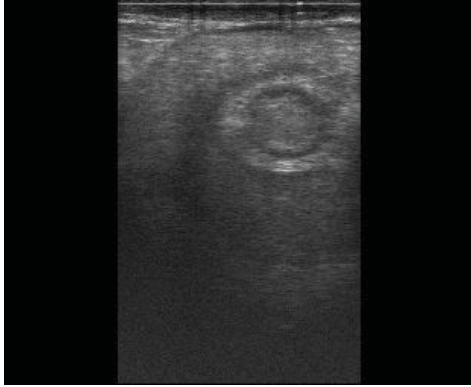


Figure 1. Ultrasonographic image of colonic faecolith.



Figure 2. Faecolith *in situ*.



Figure 3. Faeces passed even before closure of laparotomy wound.

DYSTOCIA DUE TO EMPHYSEMATOUS FETUS AND FETAL MALDISPOSITION IN A BUFFALO

Pravesh Kumar*, Amit Sharma, Madhumeet Singh, Pankaj Sood and Jitender Agarwal

ABSTRACT

A non-descript buffalo suffering with dystocia due to emphysematous fetus and fetal maldisposition and its successful management with laparohysterotomy has been discussed.

Keywords: buffaloes, *Bubalus bubalis*, dystocia, emphysematous fetus, fetal maldisposition

INTRODUCTION

Fetal emphysema is a frequent complication of parturition and a primary cause of dystocia in farm animals (Arthur *et al.*, 2001). There is putrefaction characterized by formation of gases in the subcutaneous tissue within 24-72 h subsequent to the death of the foetus and the foetus becomes soft, decomposed and distended with gases (Sane *et al.*, 1994). Srinivas *et al.* (2007) reported that 40.84 percent of dystocia in graded Murrah buffalo are due to fetal cause, among which head deviations were 42.22 percent. Amongst different reasons, the deviation of head and neck of fetus in anterior presentation are most common (Roberts, 1971) and may be in any direction (Das *et al.*, 2009). The lateral deviation of head especially in a dead fetus becomes life threatening for the dam due to uterine

contractions in inappropriately treated cases (Sane *et al.*, 1994).

The present communication describes a case of dystocia due to lateral deviation of head, further complicated by fetal emphysema in a she buffalo.

CASE HISTORY AND CLINICAL OBSERVATION

A non-descript buffalo in second lactation aged about 7 years was presented in the veterinary clinical complex of the college with the history of dystocia for the previous 2 days. The gestation length was 305 days. The buffalo had previously been treated by a local veterinary pharmacist for almost for 4 h and then the buffalo was referred to the clinics. The water bags had already ruptured one day before.

On general examination buffalo appeared dull and depressed. Feed and water intake had reduced. Udder engorgement and relaxation of sacrosciatic ligament were evident.

Per-vaginal examination revealed a dead foul smelling fetus with emphysema in anterior longitudinal presentation, dorso-sacral position with left lateral deviation of head and neck. The cervix was fully dilated. The uterine cavity was dry

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and was devoid of any lubrication. Alopecia and emphysema of the fetus were present. The case was diagnosed as dystocia due emphysematous fetus with left lateral deviation of head and neck.

TREATMENT

As the fetus was emphysematous and foul smelling and the uterine mucus membrane was edematous, it was decided to perform laparohysterotomy. It was done with standard procedure as described by Robert, 1971 by giving oblique incision parallel to milk vein. A dead male emphysemated fetus (Figure 1) was removed out from the gravid horn. Following laparohysterotomy the buffalo was treated with injection Streptopenicillin 2.5 gm b.i.d for 5 days by I.M. route, inj. Ringer's lactate 5 liters as I.V. infusion for 3 days and injection Meloxicam 25 ml I.M for 5 days. Inj. Calcium borogluconate 450 ml (300 ml slow I.V. and 150 ml S.C.) was given only once. Inj. Metronidazole was given 20 mg/kg body weight in divided doses for 5 days. The buffalo was discharged after 5 days with uneventful recovery.

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Figure 1. Emphysematous male fetus removed following laparohysterotomy.

EFFICACY OF DELTAMETHRIN AGAINST LICE INFESTATION IN BUFFALO CALVES

K. Arunachalam¹, M. Raman, T.J. Harikrishnan and T. Anna**ABSTRACT**

During the present survey, 20 buffalo calves were examined for lice infestation at a private buffalo farm, situated in Lathuwadi, Namakkal, Tamil Nadu, India. One hundred percent of the buffalo calves were infested with *Haematopinus* spp. Clinical signs included alopecia, pruritis, small crusts and scabs. Lesions were confined to the neck, shoulders and rump. Skin scrapings were examined and eggs, larva, nymphs and adult mites were recovered. The relationship between sex and age of animal was also determined; it was 94.1% in females for *Haematopinus* spp., while in males it was 80 percent. It was significantly ($P<0.05$) higher in age group of more than six months as compared to 14.2% in lesser than six months old.

Keywords: buffalo calves, age and sex, lice, prevalence, deltamethrin

INTRODUCTION

Ectoparasitic infestations cause nuisance and ill health in all livestock in addition to production loss. Besides this, they transmit many protozoan parasites. The incidence of ectoparasitic infestation in domestic animals has been reported by several workers. However, reports of louse

infestation in buffalo calves appears to be scanty. The present paper reports the incidence of louse infestation and efficacy of deltamethrin (Butox) against lice in buffalo calves maintained under a stall fed system of management at Lathuwadi village near Namakkal, Tamil Nadu.

MATERIALS AND METHODS

Twenty stall fed buffalo calves less than one year old at Lathuwadi, Namakkal, Tamil Nadu with loss of hair, thickened and rough skin, irritation, itching, emaciation formed the material for the present study. Clinical manifestations included alopecia, pruritis, and small crusts, and in addition, scab formation in one animal. General clinical examination revealed no apparent changes in body temperature or pulse rates, but all were dull and weak. Lesions were seen all over the body, particularly confined to shoulders and rump. The animals were restless and continuously rubbing their bodies against walls and bars. Examination of calves revealed the presence of various stages of ecto parasites viz., nit, nymph and adult lice on the scapular region, back, groin and axilla. Lice were collected in 70 percent ethyl alcohol and brought to the Department of Veterinary Parasitology, Veterinary College and Research Institute, Namakkal, for identification by standard

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techniques (Soulsby, 1982). Ten of the infested animals were sprayed with deltamethrin (Butox) at the rate of 2 ml / liter of water with the help of sprayer all over the body and then the remaining ten kept as untreated control group followed by chlorphenaramine maleate. Simultaneously animals were supplemented with oral liver tonic and mineral mixture daily for one week. The efficacy of drug was assessed on the basis of the presence of lice at different intervals after 0, 4, 7 and 10 days of post application.

RESULTS AND DISCUSSION

All (20) the buffalo calves were infested with various stages of lice. Infested calves manifested dullness, alopecia, anorexia, lacrimation, scratching of the body and tail against sharp object, and mild itching. The visible mucous membranes were pale and anaemic. On physical examination, various stages of lice, viz., nit, nymph and adult were found attached mainly on the areas of neck, shoulder, back region and switch of the tail.

On microscopical examination, based on the standard morphological features only one genus of louse was observed from the buffalo calves and was identified as *Haematopinus tuberculatus* (Soulsby, 1982). The infested animals were treated with deltamethrin-2% (Butox) as a spray. The calves treated with deltamethrin revealed that all the lice including developing stages died within 2 h after application. The insecticide / acaricide did not show any adverse reaction in any of the treated calves and was found safe for handlers.

On post treatment examination, animals were free from louse infestation after 24 h post treatment. Animals showed relief from the symptom of itching. The second application of the treatment

was given to control reinfestation with lice on the treated animals. Reinfestation might have occurred due to the emergence of developing nymphal stages from some of the nits sticking to the hairs and possible body contact with other infested animals in the host.

Louse infestation in buffalo calves was also recorded and reported by Chaudhury and Kumar (1961) and Sanjay and Prasad (2004) reported 33.22% overall prevalence of lice in India. Rawat *et al.* (1992) reported that out of 373 buffaloes, 60.58% were found infested with *H. tuberculatus*. The high prevalence rate of *Haematopinus* spp. recorded during the present survey may be due to lower resistance of the hosts to this parasite. Sanjay and Prasad (2004) reported that cattle and buffaloes up to 1 year of age were found to have a higher degree of ectoparasite infestation than the animals aged over 2 years which corroborated the present findings.

Many of the synthetic pyrethroids have been used for the treatment of ecto parasites in livestock and have been proved to be safe and efficient. In the present study, all the buffalo calves treated with deltamethrin spray were found to be free of ecto parasites up to 4 weeks post treatment. However, the residual effect was seen in some animals up to six weeks treated with deltamethrin. The prolonged effect of deltamethrin observed in the present study corroborated with observations reported by Singh *et al.* (1993).

It is generally observed that younger animals are more susceptible to ectoparasitic infestation as compared to adults. According to the results of the present study, some louse species were more prevalent in younger animals than older animals. This may be explained on the basis that when the animals are younger, their resistance is low and as resistance increases with the age of the

animals and then infestation decreases as the age of the animal increases. Finally, the owner was advised to separate infested and treated buffaloes from uninfested for 9 days (Radostits *et al.*, 2000) to prevent spreading of infection. Complete recovery with normal feeding habits were observed in all the infested animal after repeated treatment at a 7 day interval as this is necessary to kill the nymphal stage lice that have hatched from the eggs in the meantime. No lice could be detected macroscopically on the treated animals on the 14th day. In the present study, it was observed that deltamethrin application was quite effective for the management of lice infestation in buffalo calves.

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FOLLICULAR DYNAMICS AND UTERINE STATUS AFTER SYNCHRONIZATION OF OVULATION IN EARLY POST-PARTURIENT EGYPTIAN BUFFALOES

M.M. Kandiel*, B.A. Gad, G.A. Sosa and A.I. El-Azab

ABSTRACT

The present study aimed at instigating the buffalo ovarian and uterine response to ovsynch protocol when initiated 21 days postpartum (pp). Animals were arranged into a treated (n = 3) and a control (n = 3) group. The treated group was given the first dose of GnRH on day 0 (day 21 pp) followed by PGF2 alpha on day 7 and the second dose of GnRH on day 9. All animals were allowed a daily ultrasound examination from day 1 to day 9 and then every 12 h until detection of ovulation. The ovarian response of the treated group clearly showed the organized events of follicular growth ended by ovulation in one animal (33.3%) of the treated group; the changes in the small, medium, large and total follicle population and area did not differ significantly between the two groups; luteinization of the dominant follicle following the first GnRH injection in two animals (66.6%) indicated the reliability of the ovsynch strategy to improve the ovarian function; the uterine response was clearly evidenced by increasing the uterine wall homogeneity, decreasing the uterine lumen (less than 0.3 cm) and clearing contents by day 7 - 8 in the treated group. In conclusion, the ovsynch protocol has a beneficial effect on the ovarian function and uterine involution when applied 21 days pp in buffaloes.

Keyword: buffaloes, ovary, ovsynch, postpartum, ultrasound

INTRODUCTION

Control of the interval from parturition to subsequent conception is important to optimize the reproductive rate of a species. Buffaloes are characterized by their low reproductive efficiency as expressed by long calving interval; exceeding 500 days (Merty *et al.*, 1994), mostly due to the lowered ovarian activity (El-Wishy, 2007). In general, the ovarian response of buffaloes to stimulatory treatment seemed to be less than that reported in cattle (Totey *et al.*, 1991; Singh *et al.*, 2000). In buffaloes, the lowered availability of anovulatory follicle results in producing few and poor quality embryos (Madan *et al.*, 1996 and Misra, *et al.*, 1988).

In dairy cows, the postpartum anovulatory anestrus in dairy cows was attributed not only to a lack of follicular development, but also to the failure of a dominant follicle to ovulate (Roche *et al.*, 2000); usage of GnRH in a single injection causes an increase in the LH surge and ovulation during postpartum between days 10 - 18 in dairy (Schallenberger *et al.*, 1984) and days 21 - 31 in beef (Troxel and Kesler, 1984) cattle. A three injection schedule (GnRH-PGF2 α -GnRH),

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named Ovsynch, was successfully implicated for synchronization of ovulation in cattle (Pursley *et al.* (1995) and buffaloes (Paul and Prakash, 2005).

In the mean time of approving that the ovsynch protocol effectively induces ovulation in dairy cows as early as 21 days postpartum (Amaya-Montoya *et al.*, 2007), there is no available information on its usage during an early postpartum stage in buffaloes. The present study was designed to evaluate the ovarian response and uterine changes following synchronization of ovulation by ovsynch protocol applied earlier after calving as a point of economic value in promoting the productive and reproductive potentials of buffaloes.

MATERIALS AND METHODS

The present study was carried out on a total number of six newly parturient, lactating buffalo cows, kept in the Educational Farm, Faculty of Veterinary Medicine, Benha University during the period from April to August 2009. All animals were housed in a free-stall barn, offered 4 kg of mixed ration for lactation (consisting of cotton seed cack, line seed cack, yellow corn, bran, molasses, lime and NaCl) once daily, and suckled twice daily (0600 and 1800 h), and the average milk yield was approximately 2 - 3 kg/day.

All animals were examined ultrasonographically before the start of treatment for identifying the state of ovulation and presence of corpus luteum on the ovary. Animals were randomly allocated in two nearly similar groups for treatment and control, each of which was three buffalo cows.

Animals in the treated group (n = 3)

were treated with the ovsynch protocol (Figure 1) according to Pursley *et al.* (1995); all animals received 20 µg i.m. injection of Buserelin acetate as GnRH analogue (Receptal®, Intervet International B.V.; equal 5 ml) on day 0 (day 21 PP) followed by 500 µg i.m. injection of synthetic PGF2α; Cloprostenol (Estrumate®; Schering-Plough Animal health, equal 2 ml) on day 7 then by second i.m. injection of 20 µg GnRH analogue on day 9. In the control group, all animals were injected by saline comparable to the dose and time of the hormonal treatment.

By using transrectal ultrasonography (PieMedical 240, 6-8 MHz linear array probe), ovarian morphology was monitored daily starting from the day before the first to the second GnRH injection thence after twice daily (08.00 and 20.00 h) till occurrence of ovulation and re-examined after 10 days to confirm ovulation and occurrence of the CL. To analyze the changes in follicular dynamics after GnRH treatment, all antral follicles (≥ 2 mm) were counted and measured. The observed follicles were classified into small (< 0.5 cm), medium (0.5 - 1.0 cm) and large (≥ 1.0 cm) sizes. Diameters and volumes (cm^3) of luteal structures and cavities were determined. The transverse diameter of the anterior 1/3 section of both uterine horns and the progression of uterine involution were evaluated. Animals were observed twice daily for at least 30 minutes before milking by an experienced person to detect signs of estrus and to be bred naturally (Figure 2).

The obtained data was tabulated and statistically computed, where appropriate, by the linear regression analysis using a Microsoft Excel computer program according to Awasthi *et al.* (2006).

RESULTS

1. Ovarian findings

1.1. Follicular dynamics

As shown in Figures (3, 4, 5 and 6), the changes in the number and area of small, medium and large follicular size (<0.5 cm, 0.5-1.0 cm and >1.0 cm diameter, respectively) and whole follicular population in the treated and control groups did not show any significant variation along the experiment period. Moreover, the characteristics of follicular waves (Table 1) showed a similar pattern in the two groups except for the diameter of the dominant follicle at the first GnRH treatment, which was significantly larger prior to luteinization (1.38 ± 0.12 Vs 0.98 ± 0.06 cm, $P < 0.05$).

1.2. Ovulatory response

As shown from Figures 7 and 8, there was an occurrence of luteinization of the dominant follicle (LF) after the first GnRH injection in two out of three treated animals (66.67%) and failure of ovulation or CL development. luteinization of the largest follicle was evident ultrasonographically in two responding buffaloes by thickening in the wall (1.05 cm width) and increasing the echogenicity of the follicular wall one day after the first GnRH treatment, reaching its maximum diameter (2.69 cm) on day 2; regression of the luteal structure started by decreasing the echogenicity, the diameter and collapsing the cavity area from day 3, and became ultrasonographically difficult to detect by day 5-6 before PGF2 α treatment. Out of three treated animals, there was one buffalo cow showed (33.3%) and ovulation 24 - 36 h after the second GnRH injection.

2. Uterine findings

The dorsal uterine diameter (Figure 9A)

did not differ between the treated ($y = -0.05x + 2.90$, $R^2 = 0.69$) and control ($y = -0.06x + 2.97$, $R^2 = 0.82$) groups except on day 1 where it showed a significant decrease ($p < 0.001$) in the treated group. The ventral diameter of the uterus (Figure 9B) showed a significant ($p < 0.05$) decrease on day 4 and 7, followed by a significant ($p < 0.05$) increase on day 9 in the treated group; both groups were negatively correlated with days post-treatment ($y = -0.07x + 3.52$, $R^2 = 0.78$ and $y = -0.08x + 3.75$, $R^2 = 0.81$, respect.). The cranial diameter (Figure 9C) showed a significant increase on day 8 and 9; both treated and control groups were negatively correlated with days post-treatment ($y = -0.07x + 3.99$, $R^2 = 0.81$ and $y = -0.06x + 3.88$, $R^2 = 0.76$, respect.). The transverse diameter in the treated group (Figure 10A) showed significant ($P < 0.05$) decrease on day 1, 3, and 10, but significant ($P < 0.05$) increase on day 9 when compared to that in the control group ($y = -0.11x + 6.28$, $R^2 = 0.75$ and $y = -0.15x + 6.71$, $R^2 = 0.87$, respect.). The uterine lumen (Figure 10B) revealed highly significant ($P < 0.05$) decrease along the days of the experiment in the treated group when compared with control ($y = -0.02x + 0.45$, $R^2 = 0.84$ and $y = -0.02x + 0.59$, $R^2 = 0.73$, respect.).

DISCUSSION

The present study revealed that the ovsynch protocol applied at an early postpartum period (day 21) precisely synchronized ovulation within 24 - 36 h after the second-GnRH treatment in 1/3 (33.3%) buffaloes and induced early clearance of uterine secretion as evidenced by reducing the uterine lumen diameter. However, the changes in the total follicular population and/or area were not significantly different between the treated and the

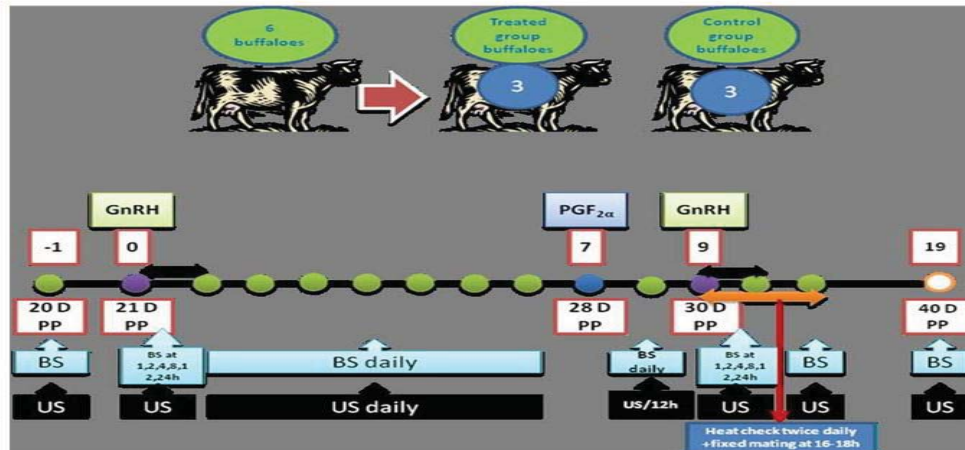


Figure 1. Diagrammatic scheme of ovsynch regimen and protocol of work. US: ultrasound examination; PP: postpartum; GnRH: gonadotrophin releasing hormone; PGF_{2α}: prostaglandin F_{2α}.

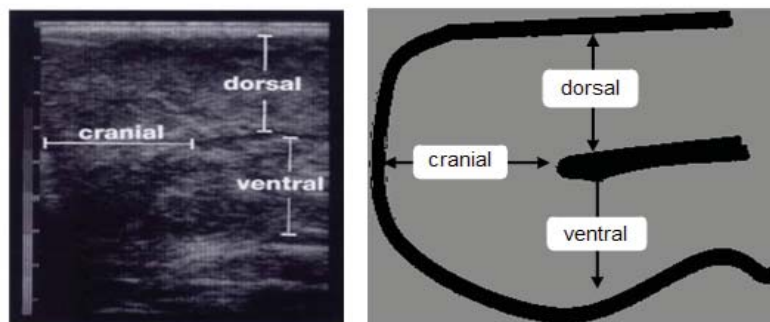


Figure 2. Ultrasonographic measuring of the dorsal, cranial, and ventral diameters of a uterine horn.

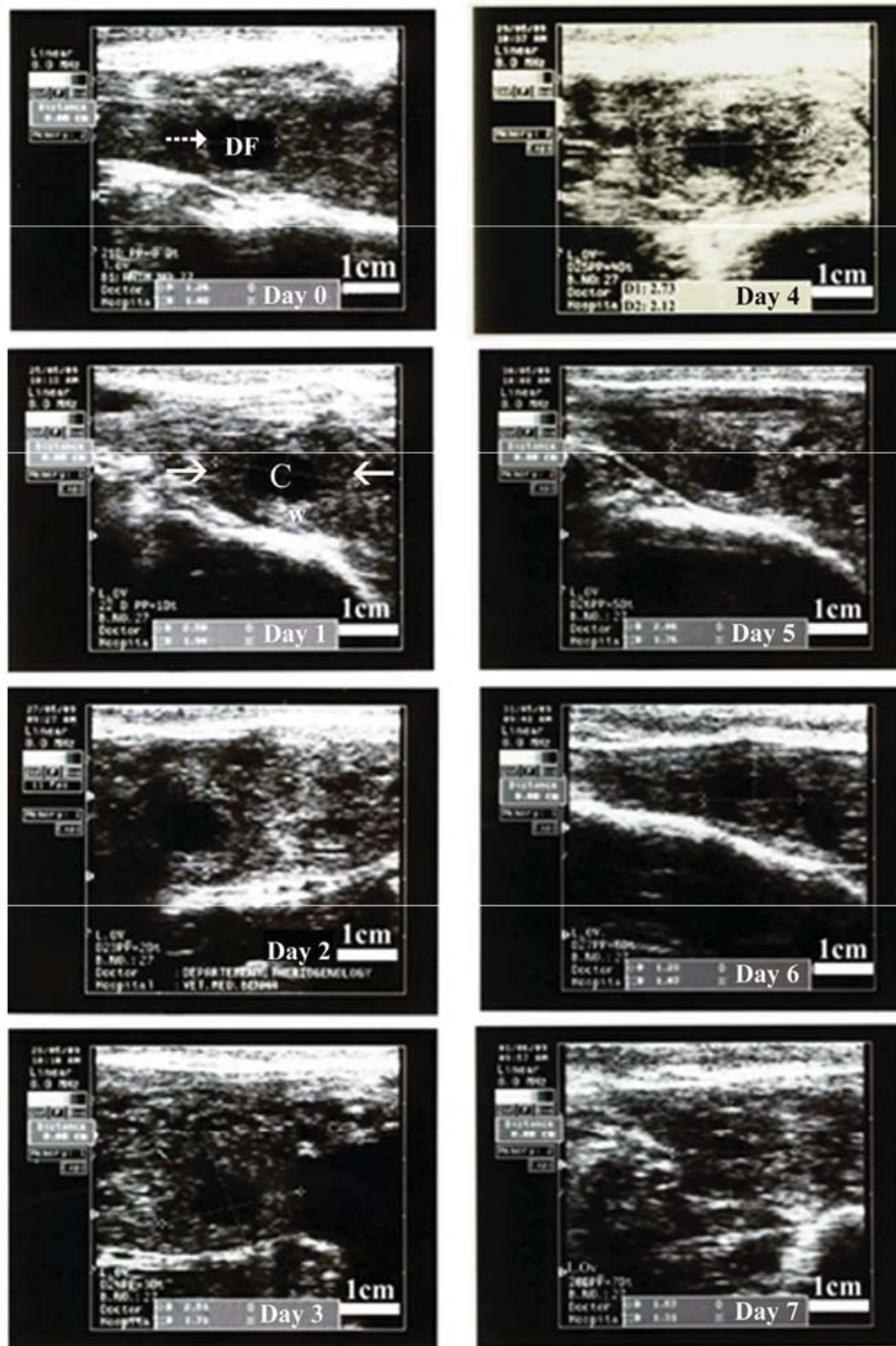


Figure 3. Changes in the characteristics of small follicle population (< 0.5 cm) in the buffaloes treated and control groups following ovsynch (GnRH-PGF2 α -GnRH) protocol started on day 21 days postpartum. Neither follicular number (A) nor area (B) differed significantly between the two groups during the monitored period. Values presented were means.

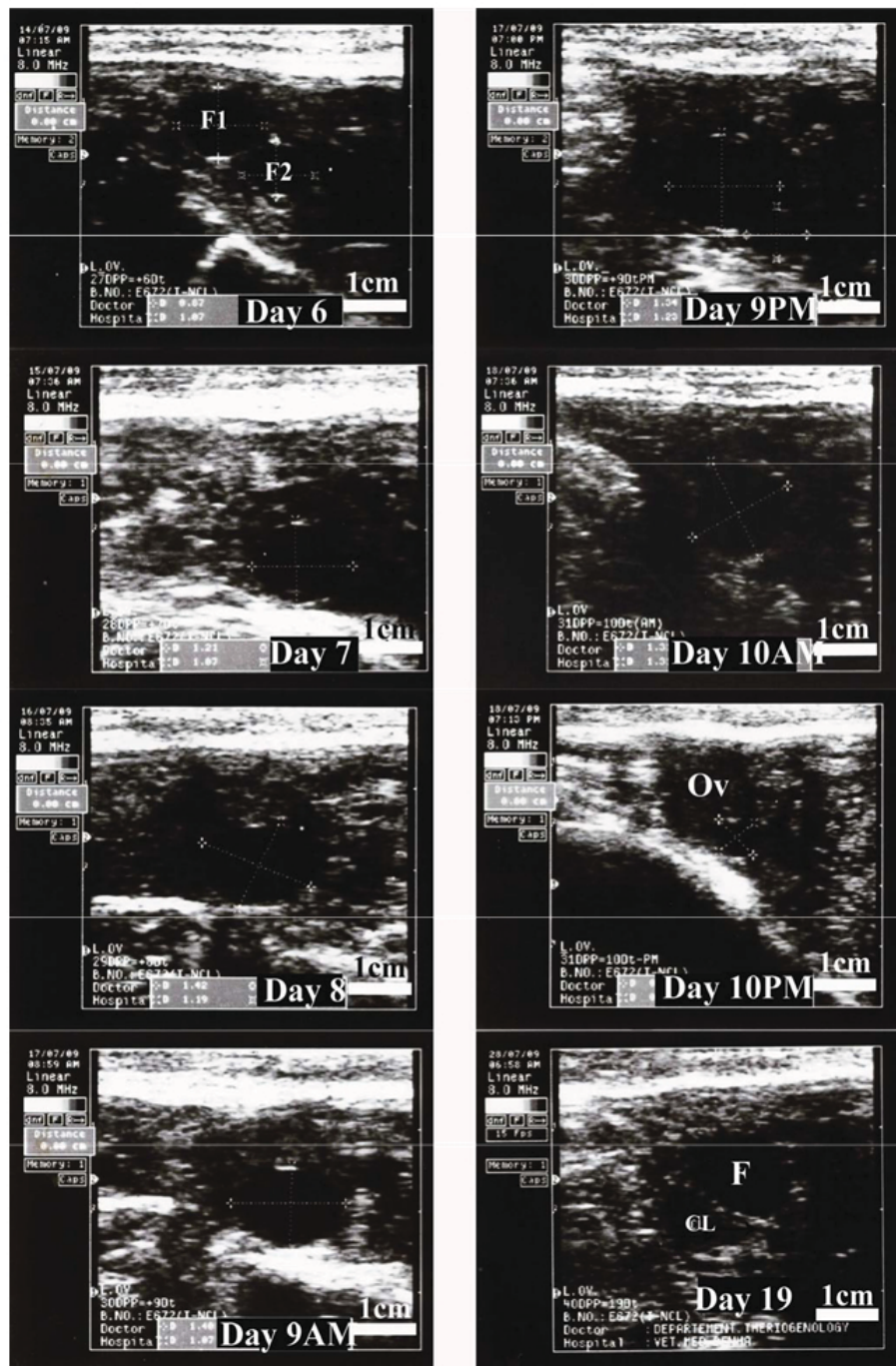


Figure 4. Changes in the characteristics of medium follicle population (0.5 - 1.0 cm) in the buffaloes treated and control groups following ovsynch (GnRH-PGF2 α -GnRH) protocol started on day 21 days postpartum. Neither follicular number (A) nor area (B) differed significantly between the two groups during the monitored period. Values presented were means.

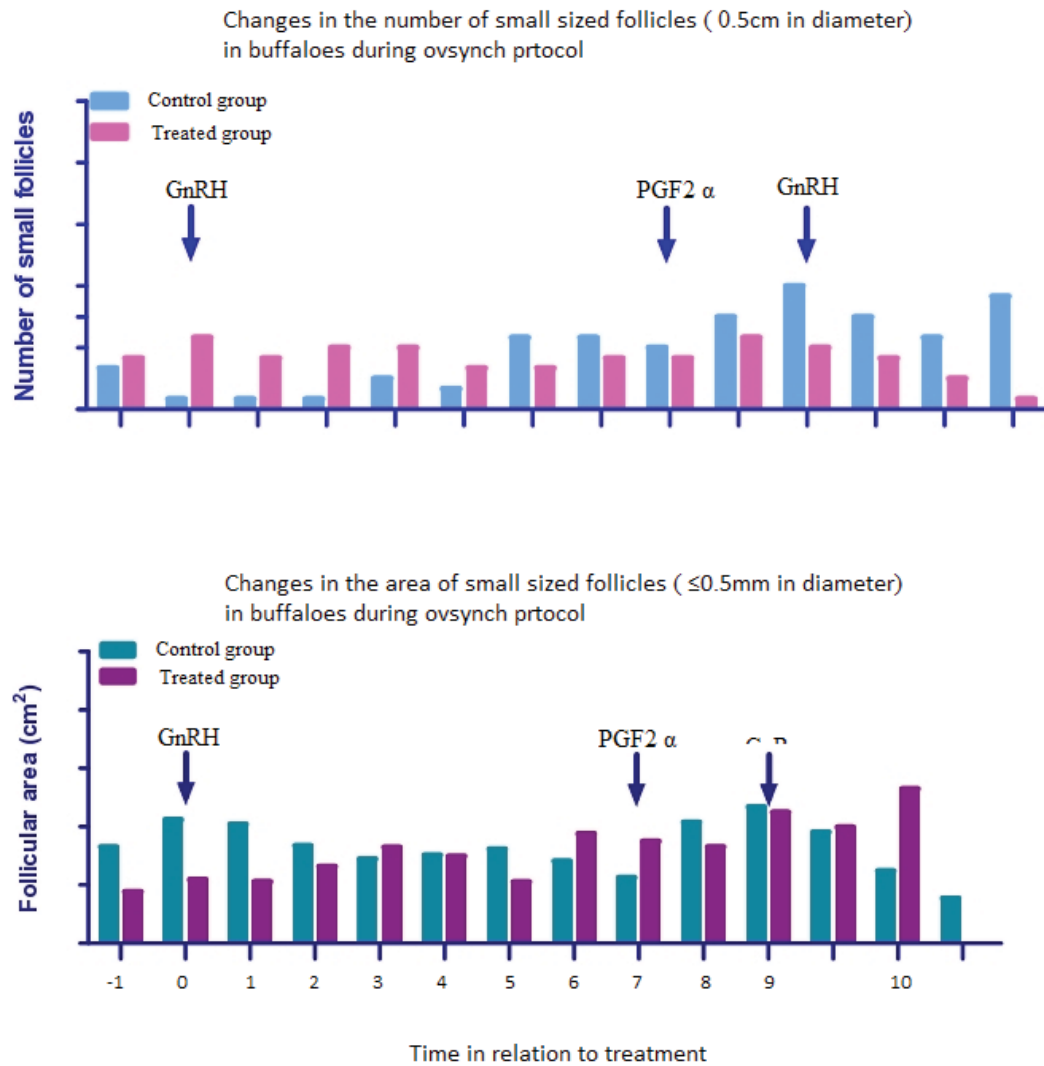


Figure 5. Changes in the characteristics of large follicle population (> 1.0 cm) in the buffaloes treated and control groups following ovsynch (GnRH-PGF2 α -GnRH) protocol started on day 21 days postpartum. Neither follicular number (A) nor area (B) differed significantly between the two groups during the monitored period. Values presented were means.

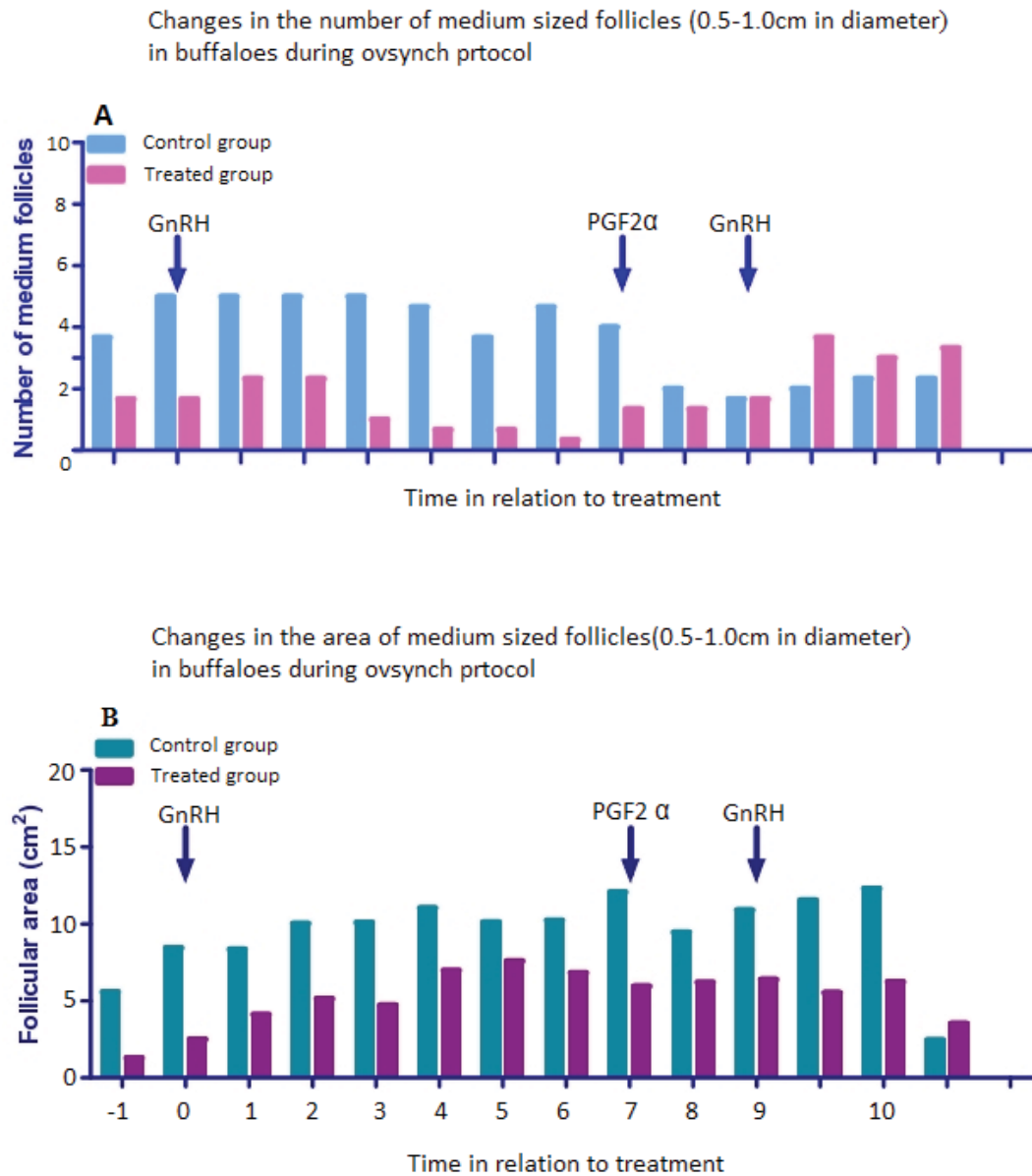


Figure 6. Changes in the characteristics of total follicle population (> 0.2cm) in the buffaloes treated and control groups following ovsynch (GnRH-PGF2 α -GnRH) protocol started on day 21 days postpartum. Neither follicular number (A) nor area (B) differed significantly between the two groups during the monitored period. Values presented were means.

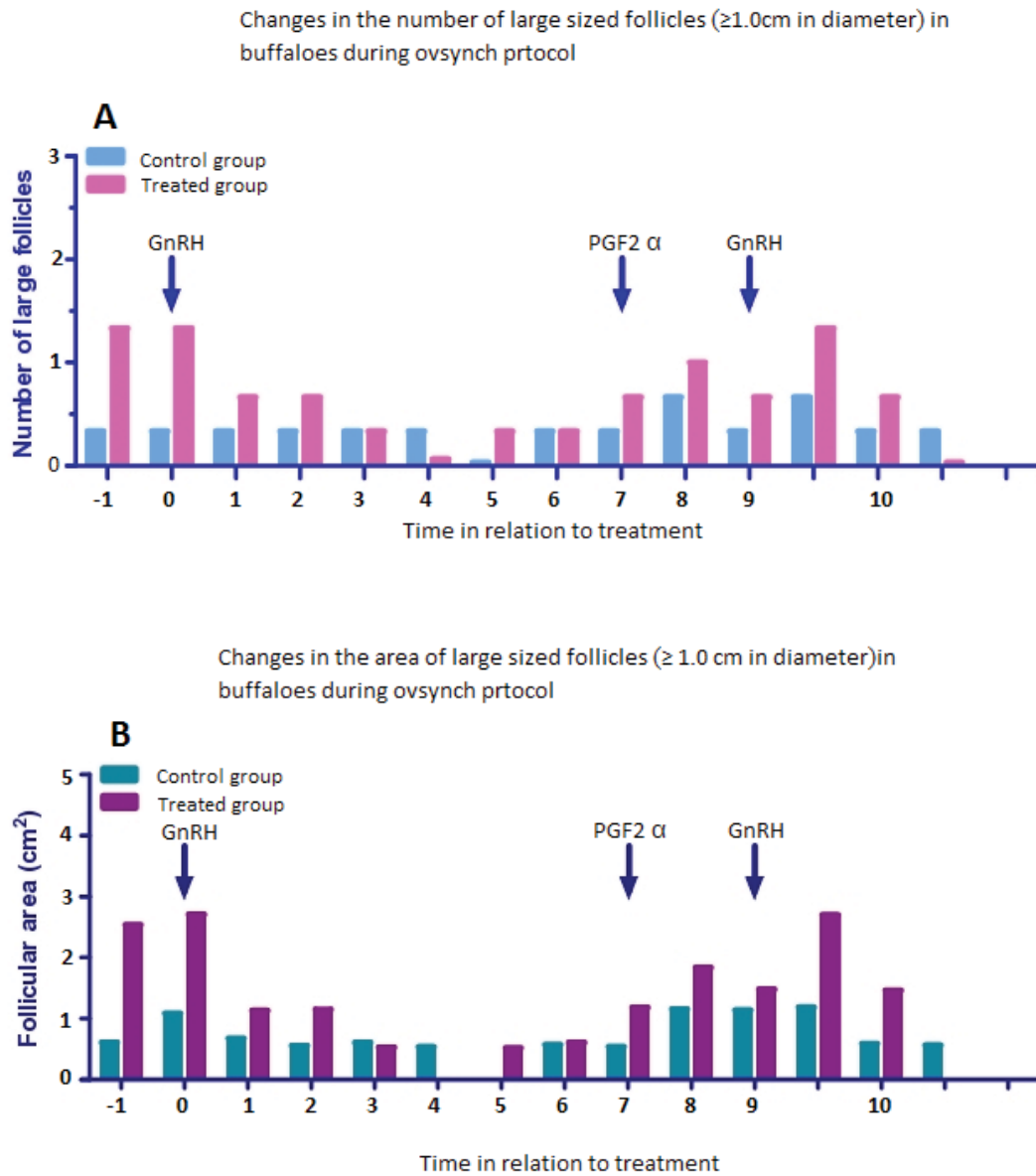


Figure 7. Representative patterns of follicular growth and regression on the ovary of buffaloes treated with ovsynch protocol 21 days postpartum. The largest follicle in the treated group showed a unique pattern of growth that ended by ovulation in one buffalo while regressed in the other two animals. Dotted and dashed lines refer to timing of GnRH and PGF2 α treatment, respectively. Lut: luteinization; Ov: ovulation.

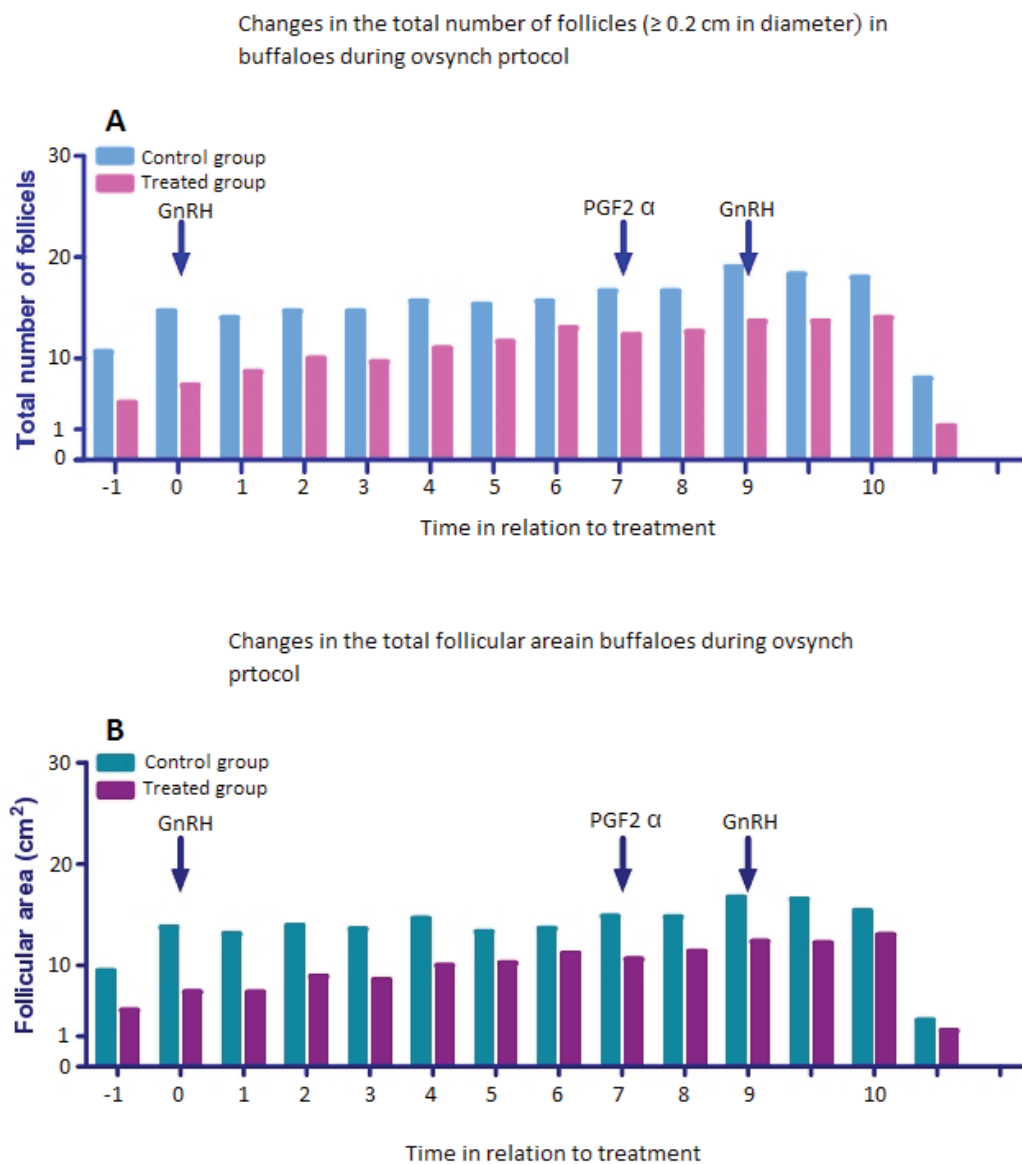


Figure 8. The pattern of induced luteal tissue development and regression on the ovary of treated buffaloes with ovsynch protocol 21days postpartum. The luteinization of dominant follicle started on Day 1. The luteal tissue volume showed a rapid decrease per unit of time as compared with luteal tissue diameter.

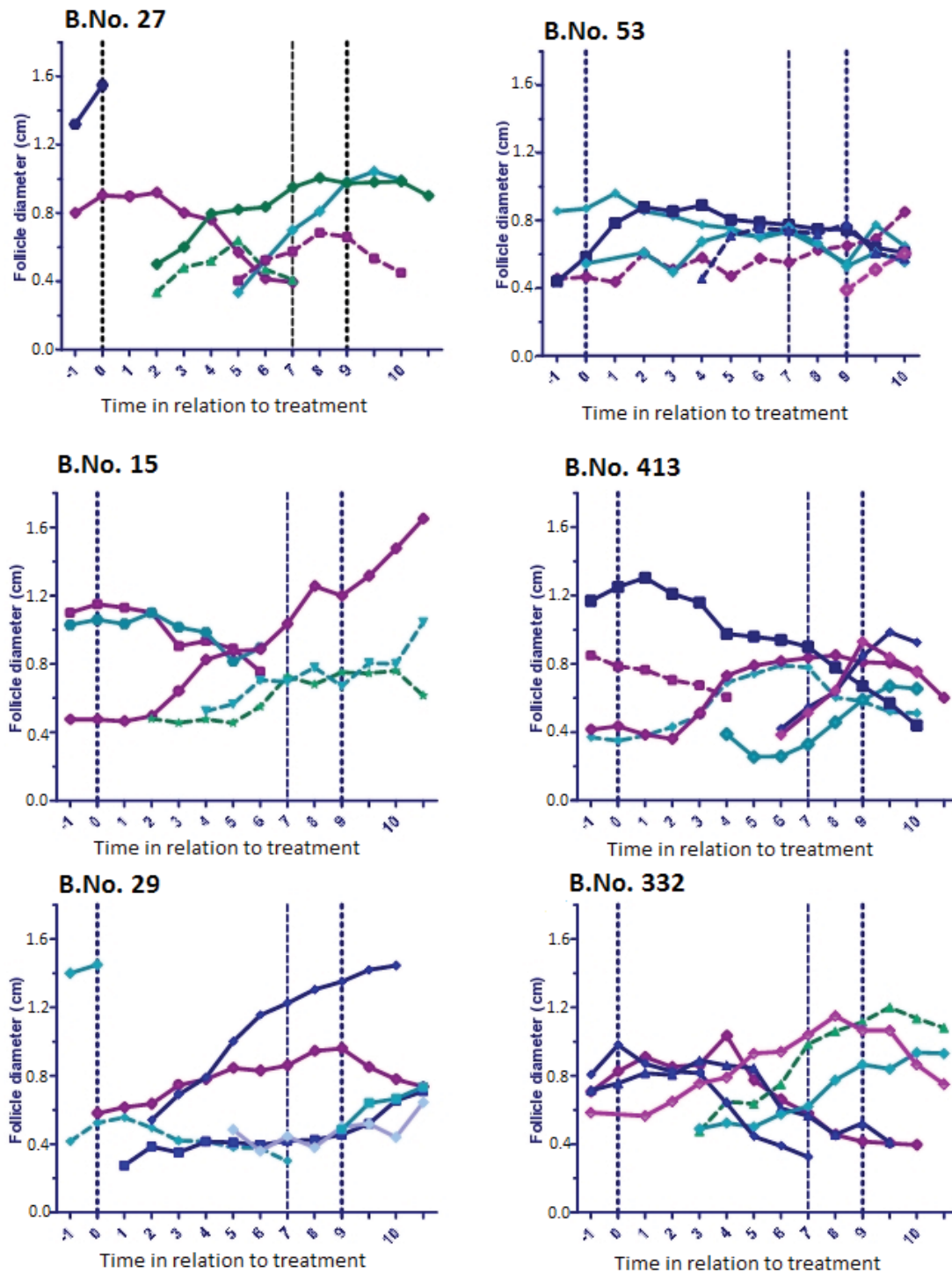


Figure 9. A representative ultrasonographic pictures of the uterus on Day 0, 2, 7, 10 in the treated (right side panel) and control (left side panel) buffaloes with ovsynch protocol started on Day 21 postpartum. The homogeneity of the uterine wall increased, while the lumen diameter and echogenicity decreased in the treated group compared with control. The arrow refers to the detectable uterine lumen in the control group on Day 10.

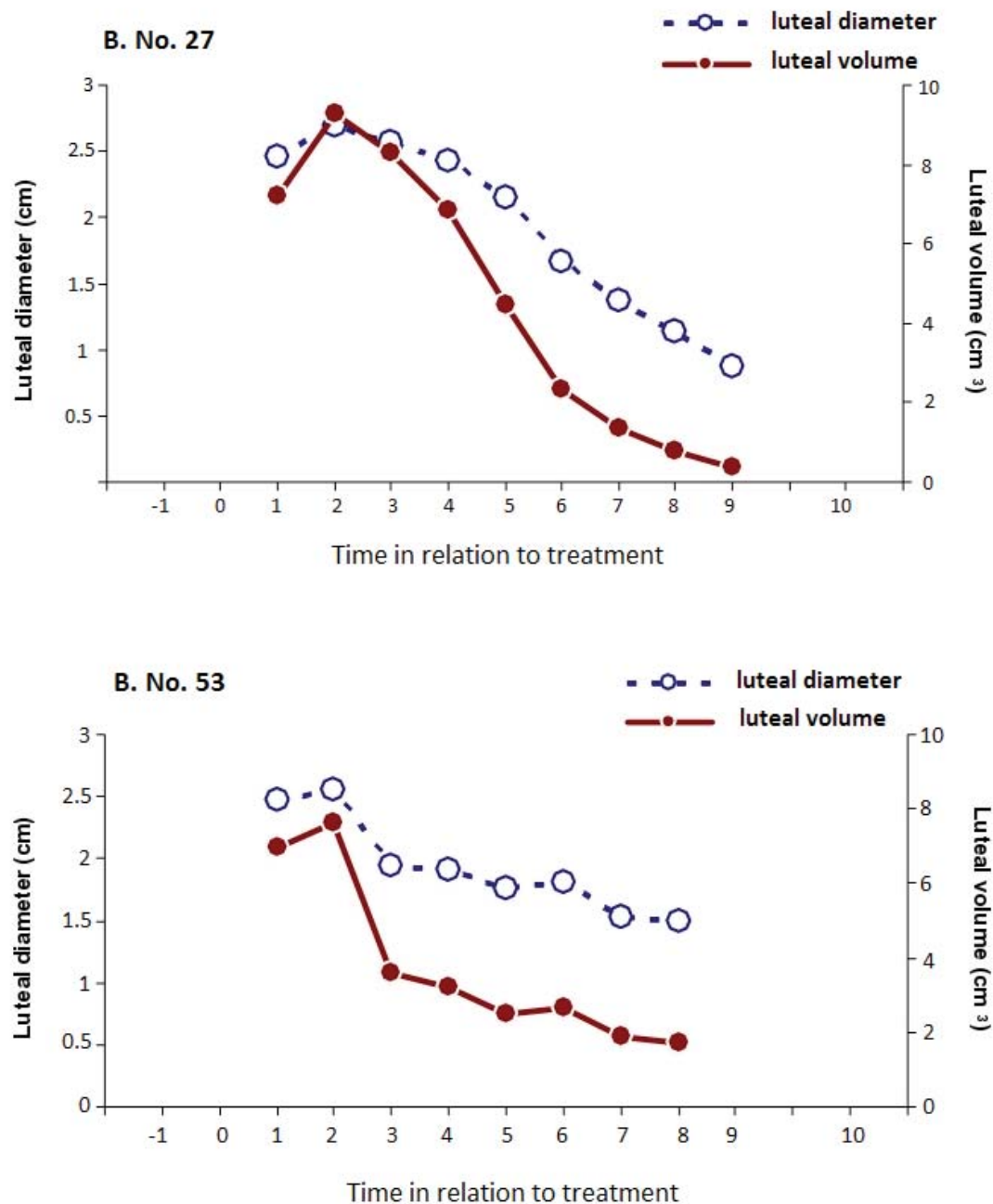


Figure 10. Changes in the uterine dorsal (A), cranial (B) and ventral diameter of the uterine wall in the treated and control groups treated with ovsynch protocol started on Day 21 postpartum. The cranial and ventral diameter of uterine horn (s) increased significantly in the treated group due to the increase in vascularity accompanying the onset of estrus. Values presented are mean \pm SEM. * $P < 0.05$, ** $P < 0.001$.

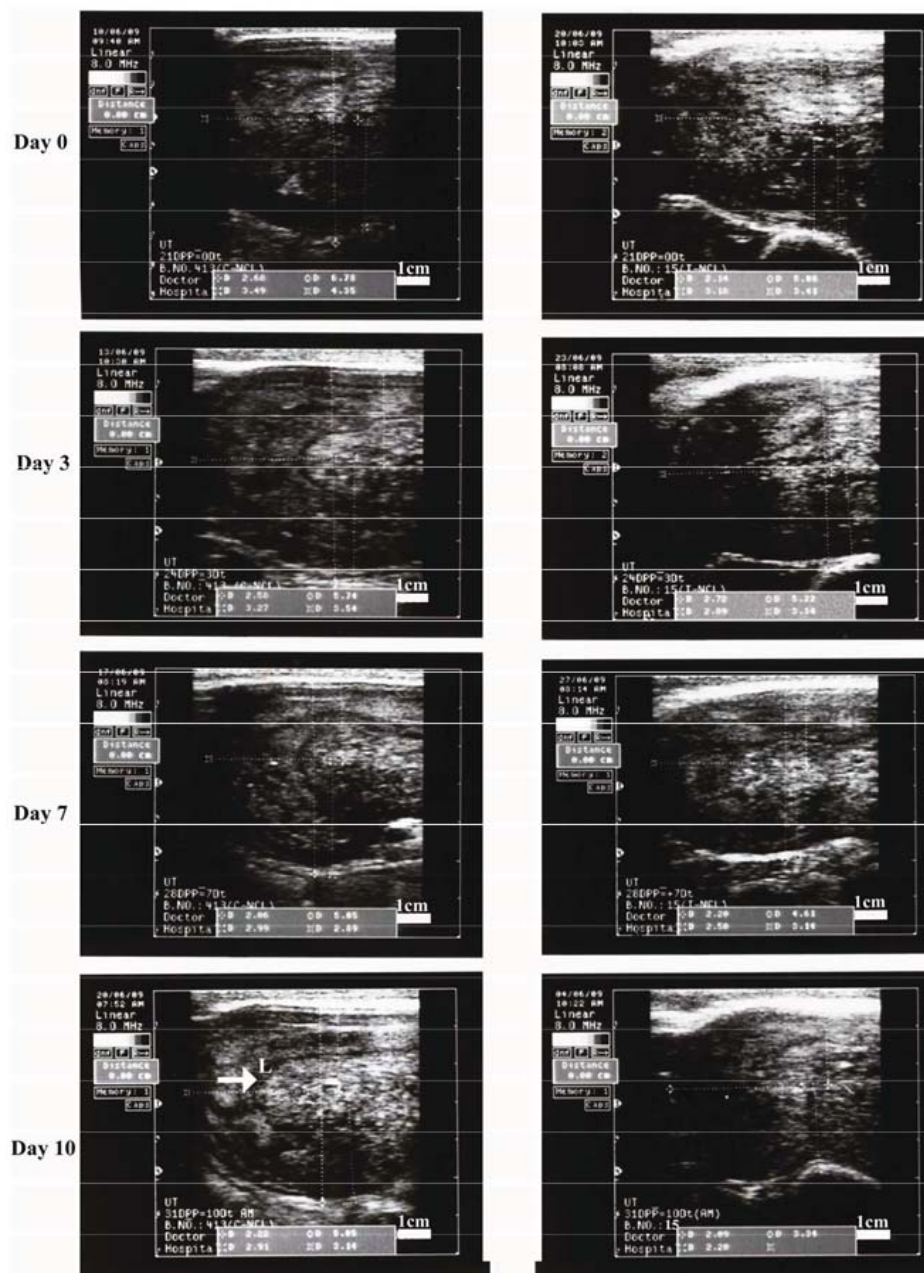


Figure 11. Changes in the transverse uterine diameter (A) and lumen (B) in the treated and control groups treated with ovsynch protocol started on Day 21 postpartum. If the diameter taken as a guide to confirm the uterine involution (< 3 cm), it did not complete neither in treated nor in control groups. If the lumen diameter used as a guide (< 0.3 cm), the uterus of treated animals involuted by Day 7 (i.e. 28 days PP). The uterine lumen showed a progressive and significant decrease in the treated animals compared with the control group. Values presented are mean \pm SEM. * $P < 0.05$, ** $P < 0.001$.

Table 1. Characteristics (Mean \pm S.E.M) of the ovarian waves in Egyptian Buffaloes treated with ovsynch during early postpartum period.

Parameters	Treated group		Control group		P-value	
	Mean	\pm SEM	Mean	\pm SEM	F-test	t-test
Diameter of dominant at start of treatment (cm)	1.38a	0.12	0.98b	0.06	0.360	0.039
Max. follicle diameter after treatment (cm)	1.38	0.18	1.01	0.10	0.462	0.139
Growth rate of the dominant follicle (mm/d)	1.49	0.07	1.65	0.17	0.243	0.453
Length of growth phase (days)	8.00	1.53	5.67	0.67	0.320	0.234
Range	5 – 10 days		5 – 7 days			
Day of emergence of the dominant follicle	2.33	1.45	2.67	2.03	0.679	0.900
Range	2 – 5 days		1 – 6 days			
Beginning diameter (cm)	0.45	0.06	0.43	0.03	0.316	0.813
Day of maximum diam. of the dominant follicle	9.33	0.33	6.33	1.45	0.100	0.114
Range	9 – 10 days		4 – 9 days			
Day of emergence of the 1st wave after treatment	2.33	0.33	4.33	0.88	0.250	0.101
Range	2 – 3 days		3 – 6 days			
Linear growth rate of the dominant follicle	0.40	0.09	0.29	0.19	0.339	0.637
max diameter of 2nd largest follicle	1.00	0.02	0.88	0.16	0.044	0.520
Day of deviation	6.67	1.20	4.33	0.67	0.471	0.165
Range	5 – 9 days		3 – 5 days			

control groups. The poor ovarian response in the treated group to the first GnRH basically might be attributed either to the little population of FSH-dependant follicles, the low LH secretion from pituitary in response to GnRH injection or the low GnRH receptors in pituitary gland. This finding came in association with some previous reports (Dufour and Roy, 1985; Lucy *et al.*, 1991; Pursley *et al.*, 1995; Walters *et al.*, 2008) indicating that the high rates of ovulation after GnRH injection are due to the presence of a potentially ovulatory follicle (> 9.0 mm); the early postpartum period is characterized by reduction in the number of small sized follicles as the number of days postpartum increases (Lucy *et al.*, 1991; El-Wishy, 2007).

The present results revealed luteinization of the dominant follicle following the first GnRH injection in the treated group, a finding which emphasized occurrence of ovulation in response to GnRH treatment during the postpartum period for 60% buffaloes (Baruselli *et al.*, 2003) and 85% cattle (Wiltbank, 1997). Follicle luteinization was detected in 37.5% non-cyclic buffaloes synchronized by ovsynch 96-118 days postpartum (Ali and Fahmy, 2007).

The present study indicated occurrence of ovulation in one case of the treated group (33.3%), 24-36 h after the second GnRH injection. This finding was consistent with that reported earlier in cows (Demüral *et al.*, 2006) and buffaloes (Warriach *et al.*, 2008) assuming that ovsynch protocol for estrus synchronization has potential application for improvement of fertility in anestrus buffaloes during early postpartum period even during extreme summer months through suppression of prolactin secretion (Roy and Prakash, 2009).

Following GnRH injection, low intensity of estrus was detected, a finding which came in accordance with some previous studies

(Pattabiraman *et al.*, 1986; Barkawi *et al.* (1995) indicating the presence of poor signs of heat in GnRH treated buffaloes.

The present study showed fluctuation in thickness of the uterus, but was negatively correlated with the day postpartum. The treated group showed a prominent clearance of the uterine lumen as indicated from the noticeable decrease of the uterine lumen echogenicity and diameter comparable to the control one. This might be attributed to the increased ovarian estradiol secretion by large follicle(s) that has a local effect to increase the rate of uterine involution (Sheldon and Dobson, 2000; Sheldon *et al.*, 2003). The ultrasonographic pattern of decreasing the uterine diameter by increasing time after parturition came in accordance with some previous studies (Okano and Tomizuka, 1987; Tian and Noakes, 1991; Kamimura *et al.*, 1993; Sheldon *et al.*, 2003). Besides, the transverse uterine diameter recorded in the present study for the treated and control groups was inconsistent with previous reports in buffaloes (Usmani *et al.*, 2001; Lohan *et al.*, 2004; Khasatiya *et al.*, 2006).

From the present study, it can be concluded that the ovsynch protocol is potentially able to improve the ovarian and uterine function when applied during early postpartum in buffaloes, a finding which needs to be confirmed in further study on a bigger number of post-parturient buffalo cows.

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EFFECT OF TCM-199 AND SYNTHETIC OVIDUCTAL FLUID (SOF) MEDIUM AND CYSTEAMINE SUPPLEMENTATION TO *IN VITRO* MATURATION MEDIA ON MATURATION, CLEAVAGE RATE AND SUBSEQUENT EMBRYONIC DEVELOPMENT OF BUFFALO OOCYTES

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A. Thangavel and G. Dhinakar Raj

ABSTRACT

The present study has been undertaken to study the effect of TCM-199 and synthetic oviductal fluid (SOF) medium and the influence of cysteamine (100 μ M) supplementation to IVM medium on maturation, cleavage and subsequent embryonic development of buffalo oocytes. In the present study it was observed that buffalo oocytes showed no significant variation in the percent of cumulus cell expansion when matured in TCM-199 and SOF media. The mean percent of cleavage rate and subsequent development to morula in buffalo oocytes matured in TCM-199 and SOF media were 28.06 ± 2.19 , 10.20 ± 0.91 and 28.99 ± 0.66 , 10.65 ± 0.40 , respectively. There was no significant difference with regard to cleavage rate and subsequent development to morula when buffalo oocytes were matured in TCM-199 and SOF media.

The percentage of oocytes that developed to morulae was significantly higher for oocytes matured in TCM-199 medium containing cysteamine (14.56 ± 1.06 vs 10.20 ± 0.91) and in SOF medium supplemented with 100 μ M of cysteamine (13.97 ± 0.80 vs 10.65 ± 0.40) compared to their respective controls whereas the cysteamine supplementation to the IVM (TCM-199) medium of buffalo oocytes insignificantly increased the cleavage rate when compared with control (33.54 ± 1.54 vs 28.06

± 2.19) and subsequent development of cleaved embryos to the morula stage (43.40 ± 2.48 vs 36.36 ± 1.65). The cysteamine supplementation to the SOF as IVM medium of buffalo oocytes also insignificantly increased the cleavage rate when compared with control (33.09 ± 1.08 vs 28.99 ± 0.66) and subsequent development of embryos to the morula stage (42.22 ± 3.12 vs 36.73 ± 1.12).

Even though the effect of cysteamine supplementation to IVM was insignificantly higher with regard to cleavage rate and development of early embryos to morulae, the percentage of oocytes that developed to morulae was significantly higher for oocytes matured in TCM-199 medium containing cysteamine (14.56 ± 1.06 vs 10.20 ± 0.91) and in SOF medium supplemented with 100 μ M of cysteamine (13.97 ± 0.80 vs 10.65 ± 0.40) compared to their respective controls, which could be attributed to cumulative effect (positive influence) of cysteamine on both the fertilization process and early embryonic development.

The beneficial effect of cysteamine on embryo development might be mediated by an increase in glutathione (GSH) synthesis. Increased oxidative stress appears to be a major factor impairing *in vitro* mammalian embryo development. The oocytes and embryos of buffaloes are more sensitive to oxidative stress because of their higher lipid content which may be a cause of (species-specific) developmental block to embryo development. The effect of

oxidative stress could be attenuated by efficient antioxidants such as GSH which scavenge reactive oxygen species (ROS). Embryos are capable of synthesizing GSH after the activation of the embryonic genome and the eight-cell block may be correlated to low intracytoplasmic concentration of GSH. Adequate amounts of GSH need to be synthesized during *in vitro* maturation of oocytes in order to support development up to the stage at which embryos acquire the biosynthetic ability.

Keywords: buffaloes, *Bubalus bubalis*, oocytes, TCM-199, synthetic oviductal fluid, SOF, embryonic development

INTRODUCTION

Oocyte maturation can be defined as those events associated with the initiation of germinal vesicle breakdown (GVBD) and completion of first meiotic division (Leibfried-Rutledge *et al.*, 1987). Maturation allows the oocyte to express its developmental potential after fertilization and is not merely confined to nuclear events or the ability to be fertilized (Gordon, 2003). The developmental competence of *in vitro* matured oocytes was significantly lower compared to oocytes matured *in vivo*, even though the nuclear maturation rates were similar, due to inappropriate maturation. Therefore maturation media have been supplemented with hormones (Salustri *et al.*, 1989), serum (Totey *et al.*, 1993), growth factors (Lonergan *et al.*, 1996) etc. in order to improve the *in vitro* developmental competence of oocytes.

MATERIALS AND METHODS

The components of various media and consumables used in these experiments were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Nunclon, Denmark, respectively unless and otherwise indicated. All the reagents were of cell culture grade. Milli Q water was autoclave sterilized and used for preparation of different media.

Ovaries from sexually mature buffaloes (*Bubalus bubalis*) were collected irrespective of age, body condition, stage of oestrous cycle and season from Chennai Corporation abattoir and utilized in this study. The ovaries were removed within 30 minutes of slaughter and washed in phosphate buffered saline (PBS) supplemented with 50 µg/ml gentamicin sulphate to remove blood and extraneous material. The washed ovaries were transported at 37°C in a thermos flask in the same media to the laboratory within 30 minutes. The extra-ovarian tissues were trimmed off and the ovaries were washed three times with tap water and five times with PBS to remove blood clots and superficial bacterial contamination. The washed ovaries were kept in a sterile beaker containing PBS supplemented with 50 µg/ml gentamycin until oocyte retrieval. Oocytes were screened under a stereozoom microscope at 10X magnification, washed thrice in 35 mm Petri dishes and graded based on their cumulus mass investment and homogeneity of ooplasm as described by Nandi *et al.* (1998).

IN VITRO MATURATION (IVM) OF OOCYTES

The oocytes were cultured *in vitro* either in synthetic oviductal fluid (SOF) medium or in TCM-

199 supplemented with 10 percent fetal bovine serum (FBS), 100 μ M cysteamine, 1.0 μ g/ml follicle stimulating hormone (FSH), 0.02 μ g/ml luteinizing hormone (LH), 1 μ g/ml 17- β estradiol and 50 μ g/ml gentamycin. IVM droplets of 100 μ l were made, overlaid with sterile mineral oil and equilibrated for 2 h in a CO₂ incubator at 38.5°C and 5 percent CO₂. Only culture grade ('A' and 'B') grade COCs were used for IVM. The COCs were rinsed three times in maturation medium and were transferred to 100 μ l of IVM droplets (15 - 20 COCs per droplet). The oocytes were allowed to mature in these droplets at 38.5°C in an atmosphere of 5 percent CO₂ in air for 24 h in a CO₂ incubator.

The maturation of oocytes was assessed based on the expansion of the cumulus mass. The degree of cumulus expansion was assessed by the morphology of the cumulus mass at the end point of *in vitro* maturation as described by Kobayashi *et al.* (1994) and Ravindranatha *et al.* (2002). Oocytes with cumulus expansion of degree 1 and 2 were considered matured. The maturation rate was expressed as percentage of the total number cultured.

Following fertilization (day 0) in BO medium (Brackett and Oliphant, 1975), oocytes were rinsed with TCM-199 and cultured for 10 days in embryo culture medium (TCM-199 with buffalo oviductal epithelial cell (BOEC) clusters) at 38.5°C in an atmosphere of 5 percent CO₂. During the course of culture, half of the embryo culture medium was replaced with fresh embryo culture medium every

48 h.

RESULTS

The percentages of buffalo oocytes that showed cumulus cell expansion in TCM-199 and SOF IVM media are presented in Table 1. Only culture-grade oocytes were used for IVM and the maturation rate was assessed based on the cumulus expansion. Out of 317 oocytes cultured in TCM-199, 292 oocytes showed cumulus expansion with a mean of 92.11 ± 0.60 percent. In SOF IVM medium, out of 296 oocytes cultured, 271 oocytes showed cumulus expansion with a mean of 91.55 ± 0.93 percent. There was no significant ($P > 0.05$) difference between TCM-199 and SOF IVM media with regard to cumulus cell expansion when the COCs were matured *in vitro*. The mean percent of cleavage rate and subsequent development to morulae in buffalo oocytes matured in TCM-199 and SOF media are presented in Table 2. The mean percent of cleavage rate and subsequent development to morulae in buffalo oocytes matured in TCM-199 and SOF media were 28.06 ± 2.19 , 10.20 ± 0.91 and 28.99 ± 0.66 , 10.65 ± 0.40 , respectively. There was no significant difference with regard to cleavage rate and subsequent development to morula when buffalo oocytes were matured in TCM-199 and SOF media

Effect of cysteamine supplementation 100

Table 1. Maturation rates of buffalo oocytes cultured in TCM-199 and SOF media.

IVM medium	No. of oocytes cultured	No. of cumulus expanded oocytes	Maturation rate Mean \pm SE	't' value (for arcsine radianse)
TCM - 199	317	292	92.11 ± 0.60	0.079 ^{NS}
SOF	296	271	91.55 ± 0.93	0.079 ^{NS}

NS- Not significant ($P > 0.05$).

Table 2. Effect of cysteamine and different maturation media on fertilization and subsequent embryonic development of buffalo oocytes (mean \pm SE).

Treatment (No. of oocytes)	Cleavage		Morulae		Blastocysts		
	(n)	% of oocytes	(n)	% of oocytes	% of 2-cell embryos	(n)	% of oocytes
TCM-199 Control (196)	55.00 \pm 0.98	28.06 ^a \pm 2.19	20.00 \pm 0.38	10.20 ^a \pm 0.91	36.36 ^a \pm 1.65	6.00 \pm 0.24	3.06 ^a \pm 0.66
SOF Control (169)	49.00 \pm 0.86	28.99 ^a \pm 0.66	18.00 \pm 0.33	10.65 ^a \pm 0.40	36.73 ^a \pm 1.12	5.00 \pm 0.15	2.96 ^a \pm 0.71
TCM-199 with cysteamine (158)	53.00 \pm 0.64	33.54 ^a \pm 1.54	23.00 \pm 0.37	14.56 ^b \pm 1.06	43.40 ^a \pm 2.48	7.00 \pm 0.28	4.43 ^a \pm 1.02
SOF with cysteamine (136)	45.00 \pm 0.39	33.09 ^a \pm 1.08	19.00 \pm 0.28	13.97 ^b \pm 0.80	42.22 ^a \pm 3.12	5.00 \pm 0.28	3.68 ^a \pm 1.22
							11.11 ^a \pm 4.03

Values within the column with different superscripts differ significantly ($P < 0.05$).

μM on cleavage was presented in Table 2. The percentage of oocytes that developed to morula was significantly higher for oocytes matured in TCM-199 medium containing cysteamine (14.56 ± 1.06 vs 10.20 ± 0.91) and in SOF medium supplemented 100 μM of cysteamine (13.97 ± 0.80 vs 10.65 ± 0.40) compared to their respective controls where as insignificantly higher percentage of cleavage rate and subsequent development of those early embryos to morula were recorded in TCM-199 medium supplemented with 100 μM of Cysteamine ($33.54 \% \pm 1.54$ and $43.40 \% \pm 2.48$ respectively) and in SOF medium supplemented 100 μM of cysteamine ($33.09 \% \pm 1.08$ and $42.22 \% \pm 3.12$) when compared to TCM -199 ($28.06 \% \pm 2.19$ and $36.36 \% \pm 1.65$) and SOF ($28.99 \% \pm 0.66$ and $36.73 \% \pm 1.12$) controls respectively.

DISCUSSION

In the present study it was observed that buffalo oocytes showed no significant variation in the percent of cumulus cell expansion when matured in TCM-199 and SOF media. Earlier report (Totey *et al.*, 1992) suggested that a significantly higher percent of buffalo oocytes maturation in TCM-199 medium when compared to SOF medium.

The mean percent of cleavage rate and subsequent development to morula in buffalo oocytes matured in TCM-199 and SOF media were 28.06 ± 2.19 , 10.20 ± 0.91 and 28.99 ± 0.66 , 10.65 ± 0.40 respectively. There was no significant difference with regard to cleavage rate and subsequent development to morula when buffalo oocytes were matured in TCM-199 and SOF media.

The percentage of oocytes that developed to morulae was significantly higher for oocytes

matured in TCM-199 medium containing cysteamine (14.56 ± 1.06 vs 10.20 ± 0.91) and in SOF medium supplemented with 100 μM of cysteamine (13.97 ± 0.80 vs 10.65 ± 0.40) compared to their respective controls whereas the cysteamine supplementation to the IVM (TCM-199) medium of buffalo oocytes insignificantly increased the cleavage rate when compared with control (33.54 ± 1.54 vs 28.06 ± 2.19) and subsequent development of embryos to the morula stage (43.40 ± 2.48 vs 36.36 ± 1.65). The cysteamine supplementation to the SOF as IVM medium of buffalo oocytes also insignificantly increased the cleavage rate when compared with control (33.09 ± 1.08 vs 28.99 ± 0.66) and subsequent development of embryos to the morula stage (42.22 ± 3.12 vs 36.73 ± 1.12).

With regard to cleavage rate this finding was contrary to Kundu (2003) but in accordance with the findings of Gasparrini *et al.* (2000 and 2003), who observed no significant effect of cysteamine on maturation and cleavage rate of buffalo oocytes even though the percent of embryos developed to the compact morula and blastocyst stage was significantly ($P < 0.05$) higher for buffalo oocytes matured in medium supplemented with cysteamine. Even though the effect of cysteamine supplementation to IVM was insignificantly higher with regard to cleavage rate and development of early embryos to morulae, the percentage of oocytes that developed to morulae was significantly higher for oocytes matured in TCM-199 medium containing cysteamine (14.56 ± 1.06 vs 10.20 ± 0.91) and in SOF medium supplemented with 100 μM of cysteamine (13.97 ± 0.80 vs 10.65 ± 0.40) compared to their respective controls, which could be attributed to cumulative effect (positive influence) of cysteamine on both the fertilization process and early embryonic development.

Increased oxidative stress appears to be a major factor impairing *in vitro* mammalian embryo

development. The oocytes and embryos of buffaloes are more sensitive to oxidative stress because of their higher lipid content which may be a cause of (species-specific) developmental block to embryo development. The effect of oxidative stress could be attenuated by efficient antioxidants such as glutathione (GSH) which scavenge reactive oxygen species (ROS). Embryos are capable of synthesizing GSH after the activation of the embryonic genome and the eight-cell block may be correlated to low intracytoplasmic concentration of GSH. Adequate amounts of GSH need to be synthesized during *in vitro* maturation of oocytes in order to support development up to the stage at which embryos acquire the biosynthetic ability. GSH also plays an important role during fertilization by contributing to the decondensation of the male pronucleus (Yoshida *et al.*, 1993).

The beneficial effect of cysteamine on embryo development might be mediated by an increase in GSH synthesis (de Matos *et al.*, 1997; Gasparri *et al.*, 2000). GSH biosynthesis depended on the availability of cysteine in the extracellular environment. Cysteamine, by reducing cystine to cysteine, elevated the levels of cysteine in the extracellular environment and thereby promoted cysteine uptake and GSH biosynthesis (Gasparri *et al.*, 2003).

The cysteamine supplementation to the medium used for *in vitro* maturation of buffalo oocytes increased glutathione synthesis in the oocyte which participated in sperm decondensation in parallel to oocyte activation and in the transformation of the fertilizing sperm head into the male pronucleus. Cysteamine supplementation might also improve subsequent embryonic development to the morula/blastocyst stage by protecting cells of early embryos from oxidative stress, promoting amino acid transport, DNA and protein synthesis and reduction

of disulphides (Yoshida *et al.*, 1993; Lafleur *et al.*, 1994 and de Matos *et al.*, 1997).

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EVALUATION OF THE SUPEROVULATORY EFFECT OF PREGNANT MARE'S SERUM GONADOTROPIN (PMSG) AND FOLLICLE STIMULATING HORMONE (FSH) IN THE NON-BREEDING SEASON OF RIVER BUFFALOES OF THE MIANKALEH PENINSULA, NORTHERN IRAN

Behrang Ekrami¹, Hamid Ghasemzadeh-Nava², Parviz Tajik² and Amin Tamadon³

ABSTRACT

Ten primiparous river buffaloes of the Miankaleh peninsula, northern Iran in the non-breeding season from the native herds were selected. The buffaloes were divided into two groups (n=5) and were injected intramuscularly with gonadotropin releasing hormone (GnRH) and a controlled internal drug-release device (CIDR) inserted on the first day. Ten days later, administration of follicle stimulating hormone (FSH) in the first group and pregnant mare's serum gonadotropin (PMSG) in the second group based on determined protocols were done. Concurrent to prostaglandin administration, CIDRs were removed and followed by estrus detection. Artificial insemination was done twice a day in both groups. Ovarian activity was assayed by ultrasonography on the first and fifth day after the start of the superovulation program. Buffaloes in which no oocyte/embryo was recovered by uterine flushing were considered non responsive to the embryo collection and were not injected with PGF_{2α}. Fifty days after insemination, pregnancy detection was performed by rectal ultrasonography. The mean (±standard deviation, SD) of follicles diameter on right ovaries in the first day in the FSH group

(9.5±1.8 mm) was less than in the PMSG group (11.1±1.1 mm, P=0.006). The mean (±SD) number of follicles on both ovaries in the fifth day in the FSH group (7.4±0.8) was significantly greater than that in the PMSG group (5.0±2.0; P=0.032). However, no oocyte/embryo was recovered in either group. Pregnancy detection 50 days after insemination showed that one cow of the FSH group and four cows of the PMSG group were pregnant. In conclusion, the PMSG and the FSH regimes induced follicular growth, but embryo recovery was poor during the non-breeding season in river buffaloes of the Miankaleh peninsula.

Keywords: pregnant mare's serum gonadotropin, follicle stimulating hormone, superovulation, non-breeding season, river buffalo

INTRODUCTION

In cattle, superovulation and embryo transfer (ET) have become established procedures for achieving genetic improvement over the past decades. However, despite the fact that the first embryo transfer in buffalo was performed 30 years ago (Drost *et al.*, 1983), the utilization of

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reproductive biotechnologies in buffalo species is still limited to experimental trials (Gasparrini, 2002; Singh *et al.*, 2009; Neglia *et al.*, 2010). Water buffaloes are notorious for their poor superovulatory responses, as commonly reported (Manik *et al.*, 1998; Techakumphu *et al.*, 2001). The ovarian response of buffaloes to superovulatory treatment has been less than one third of that reported in cattle (Carvalho *et al.*, 2002; Drost, 2007), and in those that respond, only 2-4 ovulations are induced, producing only 0-2 transferable embryos (Anwar and Ullah, 1998). The reasons for low response to superovulatory treatments in buffalo are various (Misra and Tyagi, 2007). It has been reported that the number of primordial follicles in river (Danell, 1987) and swamp (Ty *et al.*, 1994) buffaloes (30% and 20%, respectively) are lower than that described in cattle (Erickson, 1966). However, different superovulation schedules resulted in the growth of high numbers of ovulatory follicles and relatively high ovulation rates (50-70%), although embryo/ova recovery rates (13-35%) were very low (Baruselli *et al.*, 1999; Carvalho *et al.*, 2002) in the breeding season.

During the non-breeding season, September to January, reproductive function of buffaloes is reduced by increasing day length (Perera, 2011). Season affects the reproductive process of buffaloes directly through environmental temperature and photoperiod and indirectly through the quality and quantity of feed, incidence of disease and management practices (Ahmad and Noakes, 2009). Several reports suggest a lower follicular population in buffalo ovaries (Madan, 1990; Totey *et al.*, 1991). It is essential to know the number of follicles recruited in buffaloes during a superovulation program. The objective of the present study was to compare the superovulatory effect and embryo recovery rate of follicle stimulating hormone (FSH)

and pregnant mare's serum gonadotropin (PMSG) in river buffaloes of the Miankaleh peninsula, northern Iran during the non-breeding season.

MATERIALS AND METHODS

The Committee for Animal Experiments of Tehran University approved the experimental protocol. Ten primiparous river buffalo were selected during January and February, the early non-breeding season, from the native herds of Miankaleh peninsula (latitude of 50° 36' N and longitude 53° 17' E, 21-26 m below sea level), southeast of the Caspian Sea and northern Iran. The Miankaleh peninsula is located in the vicinity of the sea shore and is bush land commonly with pomegranates as the dominant vegetation. In the winter greens are scarce so the available nutrition is very weak and poor. The buffaloes of the Miankaleh peninsula graze freely on bush lands with no manual feeding. The buffaloes were milked twice daily.

Buffaloes were injected with 100 µg i.m. gonadotropin releasing hormone (GnRH, Gonabred, Parnell Laboratories, Australia). A controlled internal drug-release device (CIDR, 1.9 g progesterone, InterAg, Hamilton, New Zealand) was inserted on the first day. Ten days later, the buffaloes were randomly assigned to two groups (n=5). In the first group, follicle stimulating hormone (FSH, Folltropin-V, Bioniche Animal Health, Canada) was given i.m. twice a day (8:00 and 18:00 h) in divided doses (400 mg; 65×65, 55×55, 45×45, 35×35) and in the second group, pregnant mare's serum gonadotropin (PMSG, Folligon, Intervet, Canada) was injected 3000 IU i.m. once. Two days later, concurrent to a 250 µg i.m. prostaglandin (PGF_{2α}, Estroplan, Parnell Laboratories, Australia) injection, the CIDR was

removed. Estrus was detected every four hours in the buffalo cows using a teaser buffalo bull. Artificial insemination was done twice a day (at a 12 h interval) in both groups. Ovarian follicular status was monitored using a real-time B-mode ultrasound scanner equipped with a 7.5 MHz linear-array transducer (500 V, ami, Canada) on the first and fifth day after the start of the superovulation program. The number and diameter of anovulatory follicles were recorded.

Six days after the first insemination, uterine flushing with Dulbecco's phosphate buffered saline (Sigma, St. Louis, MO, USA), supplemented with 1% fetal calf serum (Sigma, St. Louis, MO, USA), was carried out: each uterine horn was washed with at least 500 ml of solution. Embryos were immediately searched for by filtration (Neglia *et al.*, 2003). Buffaloes from which no oocyte/embryo was recovered, were considered non responsive to the embryo collection and did not receive PGF_{2α}. Fifty days after insemination, pregnancy detection was performed by rectal ultrasonography.

Mean \pm standard deviation (SD) of the number and diameter of follicles on the first and fifth day after the beginning of the superovulation program were analyzed between two superovulatory schedules using the Mann-Whitney test. Means (\pm SD) of the numbers and diameters of follicles between the first and fifth day after the start of the superovulation program of each superovulatory schedule were analyzed by the Wilcoxon signed ranks test. (SPSS for Windows, version 11.5, SPSS Inc, Chicago, Illinois). Values of $P < 0.05$ were considered significant.

RESULTS

Two buffaloes in the FSH group showed

signs of endometritis during estrus detection and artificial insemination. The effect of FSH and PMSG administration on ovaries on different days of the superovulation programs is demonstrated in Figure 1.

The mean (\pm SD) of follicle diameter in the right ovary of the PMSG group was significantly greater than that of the FSH group in the first day ($P=0.006$; Table 1). On the fifth day, the mean (\pm SD) of number of follicles on both ovaries in the FSH group was significantly greater than that of the PMSG group ($P=0.032$). Mean (\pm SD) follicle diameters in the right ($P=0.001$), the left ($P=0.003$), and both ($P=0.004$) ovaries of the FSH group significantly increased from the first to the fifth day post treatment. However, in the PMSG group, mean (\pm SD) follicle diameter significantly increased from the first to the fifth day post treatment only in the right ($P=0.029$) and this resulted a decrease in both ($P=0.021$) ovaries. Moreover, mean (\pm SD) number of follicles on the right ($P=0.034$) and both ($P=0.039$) ovaries in the FSH group significantly increased after 4 days.

No oocyte/embryo was recovered after survey of flushing medium in either the FSH or the PMSG groups. Pregnancy detection examination showed that one buffalo cow in the FSH group and four buffalo cows in the PMSG group were pregnant.

DISCUSSION

The follicle diameter in the right ovary of the PMSG group was greater than that of the FSH group on the first day of superovulation treatment. After four days, follicle diameters in both ovaries of the FSH group increased. However, in the PMSG group, follicle diameters increased only in the right

Table 1. Mean (\pm standard deviation) of number and diameter of follicles on first day and fifth day after the start of two superovulatory schedules using follicle stimulating hormone (FSH) and pregnant mare's serum gonadotropin (PMSG) during the non-breeding season in river buffaloes (n=5) of the Miankaleh peninsula, northern Iran.

Group and day	Follicle number			Follicle diameter (mm)		
	Right ovary	Left ovary	Total	Right ovary	Left ovary	Total
FSH						
Day 1	2.2 \pm 1.3 ^a	2.4 \pm 0.8	4.6 \pm 1.9 ^a	9.5 \pm 1.8 ^{*,a}	9.9 \pm 2.8 ^a	9.7 \pm 2.3 ^a
Day 5	4.0 \pm 0.7 ^b	3.4 \pm 0.5	7.4 \pm 0.8 ^{*,b}	14.0 \pm 2.5 ^b	13.2 \pm 2.2 ^b	13.6 \pm 2.3 ^b
PMSG						
Day 1	3.0 \pm 2.0	2.6 \pm 1.6	5.6 \pm 3.5	11.1 \pm 1.1 ^{*,a}	9.6 \pm 1.5	10.4 \pm 1.5 ^a
Day 5	2.8 \pm 1.0	2.2 \pm 1.3	5.0 \pm 2.0 [*]	12.2 \pm 4.4 ^b	12.8 \pm 2.5	12.5 \pm 3.6 ^b

Stars show significant differences in each column between two superovulatory schedules ($P < 0.05$). Different superscript letters indicate significant differences in each column between the two days in each superovulatory schedule ($P < 0.05$).

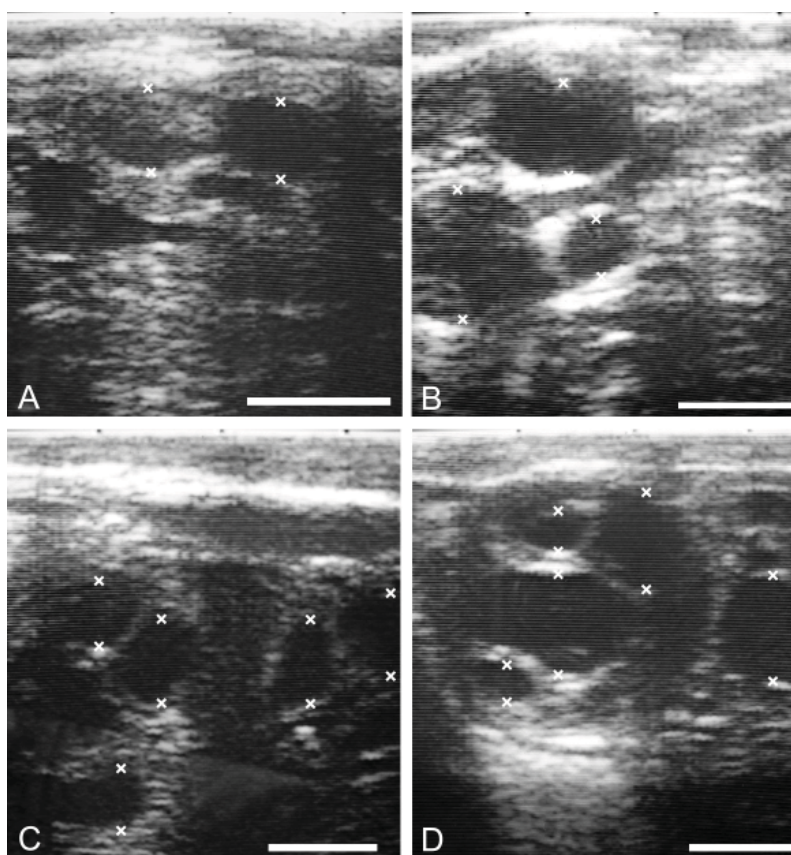


Figure 1. Sonograms (7.5 MHz) demonstrating the superovulatory response of ovaries of Miankalleh buffaloes to the PMSG (A and B) and FSH (C and D) administration (Day 5). Crosses indicate follicle or corpus luteum diameters. Line indices are 10 mm.

ovaries. Furthermore, since number of follicles on both ovaries in the FSH group increased, on the fifth day, the number of follicles on both ovaries in the FSH group was greater than that in the PMSG group. Conversely with our findings in out of reproductive season, Karaivanov (1986) reported no substantial difference in the ovarian response had been established under PMSG and FSH regimens, but the percentage of the non-ovulating follicles following the PMSG treatment was considerably higher than that following FSH treatment in the spring and summer. The percentage of the non-ovulating follicles after FSH stimulation established by Karaivanov (1986) in the summer as well as in the spring experiments (15.2 and 7.7, respectively) was lower than that reported. Patel *et al.* (2009) showed that in Toda buffaloes, late ovulation and a lower number of recruited follicles during superovulation may be the reason for low superovulation response. In the present study, recruitment of follicles in PMSG treated ovaries in out of reproductive season buffaloes was less than in FSH treated ovaries.

In the present study, no oocyte/embryo was recovered after survey of the flushing medium in either the FSH or the PMSG groups. Since no embryo was recovered, PG treatment was not done and buffaloes were left to become pregnant. One buffalo cow in the FSH group and four buffalo cows in the PMSG group became pregnant. Chantaraprateep *et al.* (1988) showed that using FSH and PMSG for superovulation in swamp buffaloes, the recovery rate of embryos was 54.5% (6/11) while the recovery rates for normal embryos, degenerated and unfertilized eggs were 37.5% (6/16) and 25% (4/16), respectively. In buffalo species, the efficiency of superovulation treatment is still poor and 1.8-2.5 embryos/donor have been reported by several authors (Misra and

Tyagi, 2007). In particular, according to Zicarelli (1997) the low number of embryos is mainly due to the low percentage of responsive subjects, which is around 55%. Zambrano-Varón and BonDurant (2007) showed failure in ovulation after the superovulation treatment with FSH in water buffalo during the non-breeding season, although the follicular recruitment and further development of ovulatory follicles was evident. Therefore, it has been supposed that the transport of ova and embryos within the oviduct may be compromised (Carvalho *et al.*, 2002). The relatively high concentration of circulating estradiol during superovulation and embryo transfer protocols in buffaloes (Beg *et al.*, 1997) may negatively affect the action of fimbriae and the recruitment of oocytes (Misra *et al.*, 1998). Moreover, Patel *et al.* (2010) observed that the total embryo recovery and viable embryo recovery were lower in buffalos with small corpora lutea. Other possible explanations that have been suggested for poor embryo recovery rates in buffalo following standard superovulation protocols include the smaller pool of primary oocytes available for recruitment, relative to bovine oocyte pools (Danell, 1987). However, given that even a smaller pool still contains tens of thousands of oocytes, this explanation by itself seems insufficient.

In conclusion, Although the PMSG and FSH regimes induced follicular growth and embryo recovery was poor, superovulation treatment with FSH caused a greater number of follicles but less pregnancy than treatment with PMSG during the non-breeding season in river buffaloes of the Miankaleh peninsula.

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MOLECULAR CHARACTERIZATION OF THE LEPTIN GENE IN RIVERINE BUFFALOES

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ABSTRACT

The present study was undertaken with the objectives of sequence characterization and studying the genetic variation in leptin gene locus 2nd exon and 3rd exon in Murrah buffalo by PCR-RFLP. The leptin gene was amplified by PCR using oligonucleotide primers standardized for *Bos taurus* species. 289 bp fragment comprising of exon 2 and 405 bp fragment containing exon 3 of leptin gene were amplified and digested with *Alu I*, *Acil*, *MspI*, *Sau3AI*, *HphI* restriction enzyme using a cattle specific primer. The sizes of the amplification products were similar in cattle and buffalo. The leptin gene in buffalo reveals monomorphism since no variation was found. The result indicates strong conservation of DNA sequence between cattle and buffalo. Nucleotide sequence variations observed in the leptin gene between *Bubalus bubalis* and *Bos taurus* species revealed 97.0% nucleotide identity. Sequence comparison of buffalo with cattle reveals variation at eight nucleotide sequences at positions 983, 1083, 1147, 1152, 1221, 1371, 3318, 3333 when all the SNPs are synonymous resulting in no change in amino acids. The milk production traits namely milk yield and fat yield could not be associated with buffalo leptin genotypes due to their monomorphic haplotype.

Keywords: leptin gene, PCR-RFLP, genetic

polymorphism, phylogenetic tree, haplotype

INTRODUCTION

Leptin is a 167-amino acid protein produced by the leptin gene (LEP), whose name is derived from the Greek word “leptos”, which means “thin”. Leptin, a 16 kD a protein that is synthesized by adipose tissue, is involved in regulation of feed intake, energy balance, fertility and immune functions (Fruhbeck *et al.*, 1998). It is one of the most useful biomolecules as a marker for identifying high performing individuals leading to better adaptability and productivity. Leptin is also responsible for the regulation of body weight and energy homeostasis (Friedman *et al.*, 1998).

Defects in leptin production cause severe hereditary obesity in animals. It has an important role in regulation of hematopoiesis, angiogenesis, wound healing, and the immune and inflammatory response. The LEP gene is the human homolog of the gene (ob) mutant in the mouse ‘obese’ phenotype (Zhang *et al.*, 1997). Since the bovine leptin gene has been identified on chromosome 4, several SNPs have been previously identified in introns and exons of leptin among different breeds of cattle. The physiological role and biology of leptin is well reviewed (Hossner, 1998 and Houseknecht *et al.*, 1998). Polymorphic studies on bovine leptin gene

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have been reported (Pomp *et al.*, 1997; Haegeman *et al.*, 2000; Lien *et al.*, 1997 and Wilkins and Davey, 1997, (Almeida *et al.*, 2003). Lagonigro *et al.* (2003); Choudhury, 2004; Yoon *et al.* (2005).

The leptin gene consists of three exons and two introns which span about 18.9 kb, of which the first exon is not transcribed into protein. The leptin gene has been mapped to chromosome 7 in human (GreGreen *et al.*, 1995) and chromosome 4 in bovine (Stone *et al.*, 1996). In buffaloes, the leptin gene is located on chromosome 8 (BBU 8q32) (Vallinato *et al.*, 2004).

The milk production trait is a quantitative trait and polygenic in inheritance. Since the milk production trait is directly related to feed intake and energy balance, it is obvious that there is an effect of leptin on milk production. Pickavance *et al.* (1998) observed that the feed intake-induced leptin increase was eliminated during lactation and they speculated that the hypoleptinemia may be an important factor promoting the hyperphagia of lactation. This data demonstrated that the onset of the negative energy balance is largely responsible for the declining leptin concentrations towards parturition and the low leptin level during lactation probably induces the hyperphagia of lactation. Plasma leptin responds to isoenergetic glucose or lipid supplementation which is dependent on the stage of lactation. Holstein cows in late lactation exhibited a robust leptin response to parenteral glucose or lipid administration, whereas early-lactation cows did not (Chelikani *et al.*, 2003). It was also reported that mammary adipocytes expressed leptin during early stages of development in sheep (Bonnet *et al.*, 2002). Leptin plays a critical role in regulating and coordinating energy metabolism (Friedman and Halaas, 1998). It regulates the metabolism of key tissues involved in the storage and dissipation of energy (Banks *et al.*, 2000).

Therefore, leptin may be important in regulating metabolic adaptation of nutrient partitioning during the energy-consuming processes of pregnancy and lactation (Moschos *et al.*, 2002).

The buffalo contributes about 54 percent of the total milk produced in India. Although the economic importance of buffaloes has always been known, yet very little work has been carried out to exploit the genetic potentials of this animal. Though studies have been carried out on characterization in cattle, similar studies in buffaloes are scarce. Association studies between leptin gene polymorphisms and live weight, energy balance, feed intake and fertility were reported in cattle (Liefers *et al.*, 2002), but little information about polymorphism and association studies of the leptin gene with phenotypic traits like milk production, fat percentage, protein yield etc. have been reported in buffaloes.

Hence the present study was undertaken with the objective of sequence characterization and identification of polymorphisms within exons 2 and 3 of the leptin gene and its association with milk and fat yield.

MATERIALS AND METHODS

Animals

The present study was conducted with 120 lactating Murrah buffaloes maintained at the cattle yard of the National Dairy Research Institute, Karnal Haryana, India.

Sample and data

About 10 ml venous blood was collected from the jugular vein of each animal into a sterile 50 ml polypropylene vial containing 0.5 M EDTA as anticoagulant.

Data on milk yield and fat percentage for the 1st, 2nd, 3rd and 4th lactations were collected from the records maintained at the cattle yard of the National Dairy Research Institute, Karnal Haryana, India.

DNA preparation

Genomic DNA was isolated from blood samples following the phenol-chloroform extraction method described by Sambrook and Russel (2001).

DNA was dissolved in TE buffer and was kept in water bath at 60°C for 2 h to dissolve the pellet properly in the buffer. The quality of the DNA was checked through spectrophotometry. DNA samples with O.D. ratios between 1.7 and 1.9 were considered as good and used for further study. The samples beyond this range were re-extracted by the phenol-chloroform extraction method. The DNA quality was also checked by running the sample in 0.8 percent agarose gel electrophoresis. The DNA samples devoid of smear were used for further study.

DNA amplification

Exon 2 and exon 3 of the leptin gene were

amplified. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique was applied to explore the polymorphism in the leptin gene.

For the amplification of the 2nd exon, 25 µl of PCR reaction mix containing 3.000 µl genomic DNA (50 ng/µl), 0.600 µl primers each (100 pM/µl), 12.5 µl Fermentas Master Mix™ (2X) and 8.3 µl double distilled H₂O. Initial denaturation at 93°C for 1 minute, denaturation at 93°C for 1 minute, annealing at 59°C for 30 seconds, extension at 72°C for 1.3 minutes were carried out for 36 cycles. The primers designed, regions amplified, annealing temperatures and product sizes are given in Table 1.

For the amplification of the 3rd exon, 25 µl of PCR reaction mix containing 3.000 µl genomic DNA (50 ng/µl), 0.600 µl primers each (100 pM/µl), 12.5 µl Fermentas Master Mix™ (2X) and 8.3 µl double distilled H₂O. Initial denaturation at 93°C for 1 minute, denaturation at 93°C for 1 minute, annealing at 56°C for 30 seconds, extension at 72°C for 1.3 minutes were carried out for 36 cycles. The primers designed, regions amplified, annealing temperatures and product sizes are given in

Table 1. Description of primers used and the amplified products of the different loci studied.

Locus	Primer sequence 5'-3'	T _m	Primer length bp	Primer source	Amplified product length
Primer I	F-5'- GGT GGT AAC GGA TCA CAT GG -3' R-5'- CCA CGG TTC TAC CTC GTC TC -3'	59 °C	20 20	D	289 bp fragment containing of exon II
Primer II	F-5'- GCA TAG CAG TCC GTC TCC TC -3' R-5'- TTC CCT GGA CTT TGG GAA G -3'	56 °C	20 19	D	405 bp fragment containing of exon III

All primers were obtained from IDT, United States of America, synthesized in 100 nM scales.

*D= Designed by using Primer 3 software.

Table 1.

RFLP and polyacrylamide gel electrophoresis

AluI, *AccI*, *HphI* restriction enzymes (0.07 µl of 10 U/ µl) and *MspI*, *Sau3AI*, *PvuII*, *HindIII*, *HinfI*, *Eco32I*, *Eco147I* and *Kpn2I* (0.14 µl of 5 U/ µl) were applied on both the contigs (20 µl of PCR product each time). The reaction mixture for RE digestion was kept for incubation at 37°C for 4 h. Horizontal electrophoresis on 2-3% agarose gel was used to resolve restriction fragments and was visualized by ethidium bromide staining. The ethidium bromide was added to the agarose gel of 1 µl/100 ml of gel. The agarose gel electrophoresis was performed in 1X buffer at 100 volts for 30, 60, and 90 minutes till complete separation of all fragments of RE digested gene fragments and PCR marker. The restriction digested gene fragments were visualized on UV transilluminator and photographed with a gel documentation system.

Nucleotide sequencing

The PCR products were concentrated to 50 ng/µl by pooling several tubes to precipitate by the isopropanol procedure. In order to obtain clean fragments for sequencing, the PCR products were separated by electrophoresis in a TAE agarose gel containing ethidium bromide using standard protocols. The desired PCR product band was excised using a clean, sterile razor blade or scalpel (bands were visualized in a medium or long wavelength (e.g., ≥ 300 nm) UV light, and excised quickly to minimize exposure of the DNA to UV light). The minimum agarose slice was transferred to a 1.5 ml microcentrifuge or screw cap tube and then purified by using commercially available gel extraction kits (Qiagen). Quantification was done by loading one µl of eluted sample in 1% agarose

gel and comparing with standard molecular markers (Phi X 174 DNA ladder or 100 bp DNA ladder). Only samples with good concentration (>50 ng/ µl) were selected and subjected to sequencing. Amplified PCR products from each set of primers were subjected to custom DNA sequencing from both ends (5' and 3' ends). Representative samples from each of the variants obtained by RFLP analysis were also custom sequenced from M/s. Chromous biotech, India.

Sequence data analysis

Sequence data were analyzed mostly by DNASTAR software. Sequence data was analyzed by using Chromas (Ver. 1.45, <http://www.technelysium.com.au/chromas.html>). Sequence data from variants of different regions were subjected to multiple alignments (DNASTAR, Clustal W) for identifying the SNPs.

Database search

The database search of sequences for a possible match to the DNA sequence of growth hormone gene was conducted using the BLAST algorithm available at the National Center for Biotechnology Information (NCBI, Bethesda, MD). Translated protein sequences of leptin genes in different species namely *Bos Taurus*, *Bos indicus*, *Ovis aries*, *Capra hircus* and *Homo sapiens* were also subjected to BLAST algorithm.

Statistical analysis

The frequencies of gene and genotypes were estimated for the identified locus as per the method suggested by Falconer and Mackay (1998). Association study with the milk production parameters with the genotype of leptin gene could not be achieved due to monomorphism of the alleles of the leptin gene in buffalo.

RESULTS AND DISCUSSION

Identification of Genotypes

The PCR amplification generated a 289 bp for exon II (Figure 1) and a 405 bp for exon III segment (Figure 2) for the leptin gene of buffalo. The bubaline leptin gene is homologous to the cattle leptin gene of similar length (Ji *et al.*, 1998), thus it indicates strong conservation of DNA sequences in both species. Nucleotide sequence organization is similar to other species, as goat, sheep, mouse, human, monkey, camel, cat, dog, donkey, elephant, horse, yak, pig and rat (www.ncbi.nlm.nih.gov).

PCR-RFLP of Leptin gene

Size of various electrophoretic bands observed by PCR-RFLP analysis of the leptin gene with various restriction enzymes in Murrah buffaloes are given in Table 2.

In the present study, PCR-RFLP analysis using all the eleven enzymes did not reveal polymorphism in either of the exons of the leptin gene in Murrah buffaloes.

Alu-I digestion of the amplified product of the 2nd exon revealed two products of 189 and 100 bp (Figure 3). Aci-I digestion of the amplified product of the 2nd exon revealed two products of 89 and 200 bp (Figure 4). *MspI* digestion of the amplified product of the 2nd exon revealed two products of 79 and 210 bp (Figure 5). *Sau3AI* digestion of the amplified product of the 2nd exon revealed two products of 89 and 200 bp (Figure 6). *HphI* digestion of the amplified product of the 2nd exon revealed two products of 89 and 200 bp (Figure 7).

Alu-I digestion of the amplified product of the 3rd exon revealed two products of 55 and 350 bp (Figure 8). Aci-I digestion of the amplified product of the 3rd exon revealed two products of 135 and

270 bp (Figure 9). *MspI* digestion of the amplified product of the 3rd exon revealed two products of 255 and 150 bp (Figure 10). *Sau3AI* digestion of the amplified product of the 3rd exon revealed two products of 105 and 300 (Figure 11). *HphI* digestion of the amplified product of the 3rd exon revealed two products of 105 and 300 bp (Figure 12).

Since the Murrah buffaloes included in the present study were found to be monomorphic, it was not feasible to analyze the data with respect to milk and fat yield (Table 3). It might be because of the fact that the animals were in a closed herd. Similar monomorphism of this gene in cattle was also observed by others in the bubaline leptin gene. Kumar *et al.* (2003) reported the absence of polymorphism within 522bp PCR product of leptin gene in buffalo digested with *HinfI* restriction enzyme. Vallinoto *et al.* (2004) amplified promoter and exon 1 with primers designed from the bovine leptin gene. Three SNPs and one microsatellite were identified. No polymorphisms were detected in exon 2. Similar monomorphism of buffalo were also reported by PCR-RFLP in growth hormone gene (Pal and Chatterjee, 2010). Therefore, this monomorphism of the buffalo may be a species specific characteristic of buffalo. Thus, the gene and genotypic frequencies were found to be 1.00.

However in cattle (*Bos taurus*), polymorphism was detected for the leptin gene by a number of researchers. Kulig *et al.* (2009) investigated how leptin gene polymorphisms affected milk production traits such as milk yield, fat and protein yield, and fat and protein content in Jersey cows. Two single-nucleotide polymorphisms (SNPs) were genotyped, using *Sau3AI* RE. RFLP polymorphisms within the bovine leptin gene were detected by using *HinfI* restriction enzyme and it was found that allele A positively affected milk production traits in Friesian cattle and they

Table 2. PCR-RFLP analysis of the leptin gene with different Res.

REs used	Contig 1 (1173-1344)			Contig 2 (3103-3462)		
	PCR product	Cutting sites	Pattern	PCR product	Cutting sites	Pattern
<i>AluI</i>	289 bp	189 & 100 bp	Monomorphic	405 bp	55 & 350 bp	Monomorphic
<i>AciI</i>	289 bp	89 & 200 bp	Monomorphic	405 bp	135 & 270 bp	Monomorphic
<i>MspI</i>	289 bp	79 & 210 bp	Monomorphic	405 bp	255 & 150 bp	Monomorphic
<i>Sau3AI</i>	289 bp	89 & 200 bp	Monomorphic	405 bp	105 & 300 bp	Monomorphic
<i>HphI</i>	289 bp	89 & 200 bp	Monomorphic	405 bp	105 & 300 bp	Monomorphic
<i>HindIII</i>	289 bp	No	-	405 bp	No	-
<i>HinfI</i>	289 bp	No	-	405 bp	No	-
<i>Eco32I</i>	289 bp	No	-	405 bp	No	-
<i>Eco147I</i>	289 bp	No	-	405 bp	No	-
<i>Kpn2I</i>	289 bp	No	-	405 bp	No	-
<i>PvuII</i>	289 bp	No	-	405 bp	No	-

Table 3. Lactation wise maximum and minimum milk and fat % in animals.

Lactation	Milk production			Fat %		
	Minimum (Kg)	Maximum (Kg)	Average (Kg)	Minimum	Maximum	Average
First	1994	3339	2089.93	6.00	10.00	7.31
Second	1922.5	3620	2294.60	5.9	10.2	7.45
Third	1915	3414.5	2107.65	5	8.2	7.48
Fourth	1713	3748	2033.00	6	8.5	7.79

indicated significant superiority of allele A over allele B for milk and milk protein yields and body conformation traits (Khaleel *et al.*, 2009).

Pannier *et al.* (2009) reported four SNP loci which were found to be in linkage disequilibrium and thus, the frequencies of each of the 16 possible haplotypes were inferred by maximum likelihood. No significant association between any individual SNP and haplotype was found with intramuscular fat values in *Bos taurus*. Fortes *et al.* (2009) have reported three genotypes in *Bos taurus* x *Bos indicus* crossbred cattle with 7.7 % higher frequency of the T allele.

Buchanan *et al.* (2003) genotyped 416 Holstein cows by using restriction enzyme Kpn21 and compared lactation performance data using a mixed model. Animals homozygous for the T allele produced more milk and had higher somatic cell count linear scores, without significantly affecting milk fat or protein percent over the entire lactation.

Dandapat *et al.* (2010) observed polymorphism using *HphI* -PCR-RFLP in *Bos taurus* x *Bos indicus* crossbred cattle exhibited AA, AV and VV genotypes with their respective frequency of 0.57, 0.36 and 0.07 and gene frequency as 0.75 and 0.25 for the A and V alleles, respectively. However, they have reported monomorphic pattern in Sahiwal cattle. Since no mutation was found in Sahiwal cattle and only the A allele was present throughout the population studied, the frequency of the A allele was 1.

Nucleotide sequencing and SNP detection

The nucleotide sequences for exon 2 and exon 3 for the bubaline leptin gene have been depicted in Figure 13 and Figure 14 respectively. The sequence obtained for the Murrah is compared and aligned with sequence of *Bubalus bubalis* using

the MegAlign program of DNASTAR software. Amplified regions of both the contigs were custom sequenced by using forward and reverse primers. The leptin gene sequences for exon 2 and exon 3 have been submitted to the gene bank. The PCR amplification procedure for two contig regions of Leptin gene has been standardized which yielded consistent and specific amplification. The leptin gene in Murrah buffaloes included in present study is monomorphic as revealed by PCR-RFLP analysis using *AluI*, *AciI*, *MspI*, *Sau3AI*, *HphI* restriction enzymes. *HindIII*, *HinfI*, *Eco32I*, *Eco147I*, *Kpn2I* and *PvuII* restriction enzymes did not reveal any cutting site in both the contigs. However, reports were available regarding the cutting site of the above enzymes in the leptin genes of cattle and other species. Sequence data were analysed using chromas (Ver.1.45, <http://www.technesium.com.au/chromas.html>).

Clustal W multiple alignments with *Bubalus bubalis* sequence revealed nucleotide changes at eight positions in the gene. These variations are found in nucleotide sequences at positions 983 in intron 1; at 1083, 1147, 1152 and 1221 in exon 2; at 1371 in intron 2; at 3318 and 3333 at exon 3 (Figures 13 and 14). Eight SNPs were discovered by studying variation at eight nucleotide sequences at positions 983, 1083, 1147, 1152, 1221, 1371, 3318, 3333 while comparing leptin gene sequences with other species. However, all these nucleotide changes are synonymous, i.e. there is no change in amino acids.

Phylogenetic analysis of buffalo with other species

The nucleotide sequences deduced for the respective exons of the leptin gene in the Murrah buffaloes were arranged to represent the coding region and were compared with other leptin



Figure 1. Resolution of PCR amplified product of Primer I on 1.5% agarose gel.
Lane 1-9 and 10-19 : PCR product (289 bp)
Lane M : 100 bp Molecular Marker

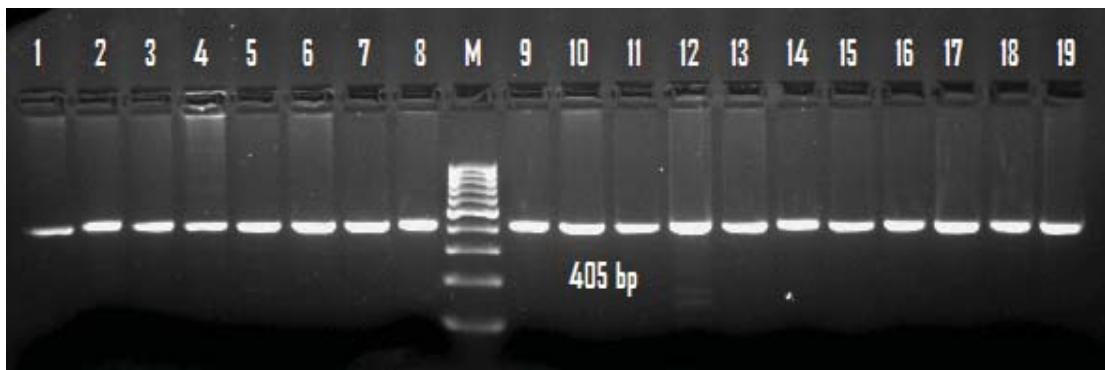


Figure 2. Resolution of PCR amplified product of Primer II on 1.5% agarose gel.
Lane 1-8 and 9-19 : PCR product (405 bp)
Lane M : 100bp Molecular Marker

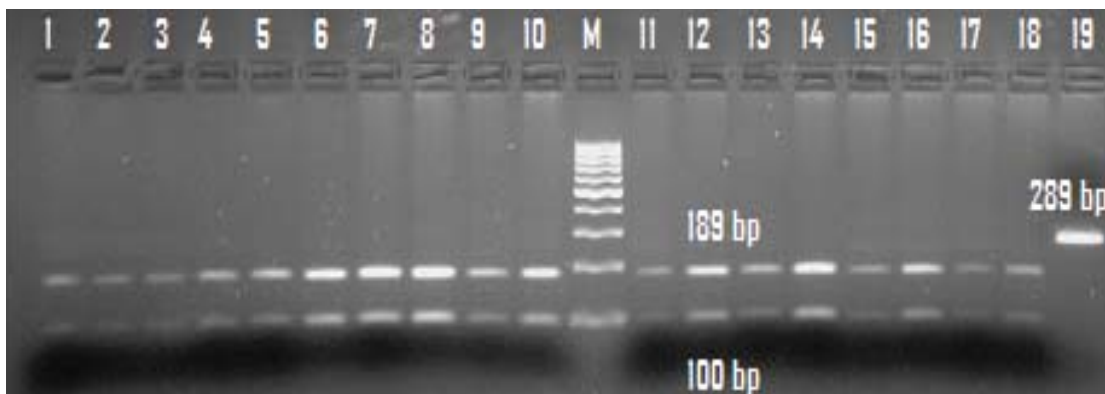


Figure 3. PCR-RFLP of primer I of the leptin gene on 2.5% agarose gel using *AluI* RE in Murrah buffaloes.
Lane 1-10 and 11-18 : 2 Bands (100 bp and 189 bp)
Lane 19 : PCR Product (289 bp)
Lane M : 100 bp Molecular Marker

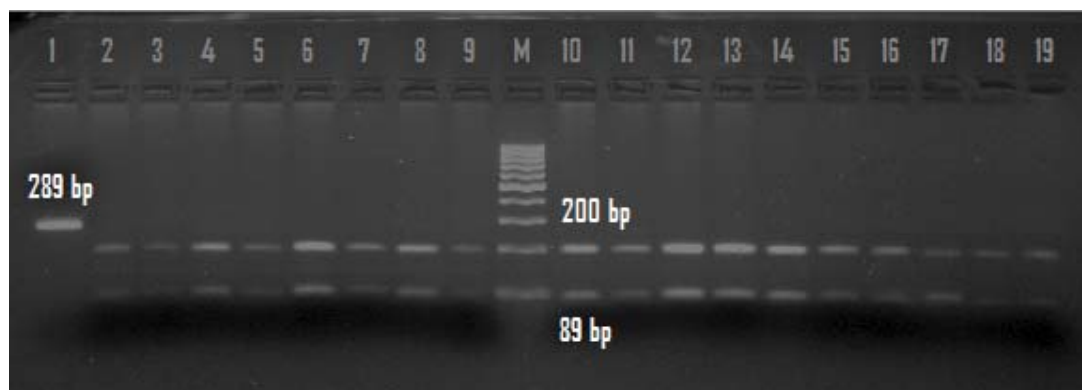


Figure 4. PCR-RFLP of primer I of the leptin gene on 2.5% agarose gel using *Acil* RE in Murrah buffaloes.

Lane 2-9 and 10-19 : 2 Bands (200 bp and 89 bp)

Lane 1 : PCR Product (289 bp)

Lane M : 100 bp Molecular Marker

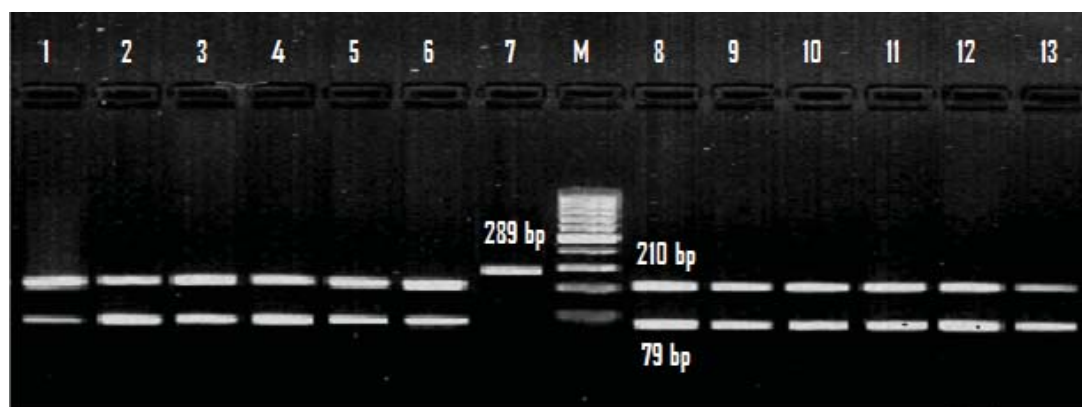


Figure 5. PCR-RFLP of primer I of the leptin gene on 2.5% agarose gel using *MspI* RE in Murrah buffaloes.

Lane 1-6 and 8-13 : 2 Bands (210 bp and 79 bp)

Lane 7 : PCR Product (289 bp)

Lane M : 100 bp Molecular Marker

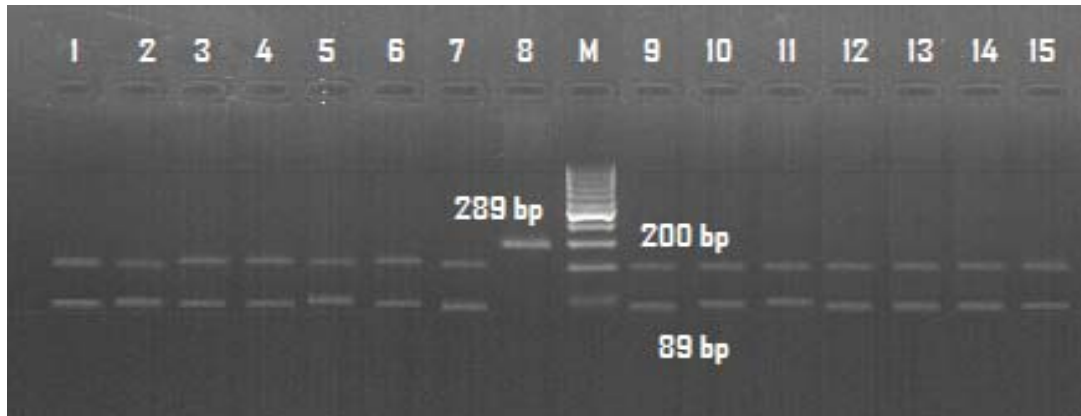


Figure 6. PCR-RFLP of primer I of the leptin gene on 2.5% agarose gel using *Sau3AI* RE in Murrah buffaloes.

Lane 1-7 and 9-15 : 2 Bands (200 bp and 89 bp)

Lane 8 : PCR Product (289 bp)

Lane M : 100 bp Molecular Marker

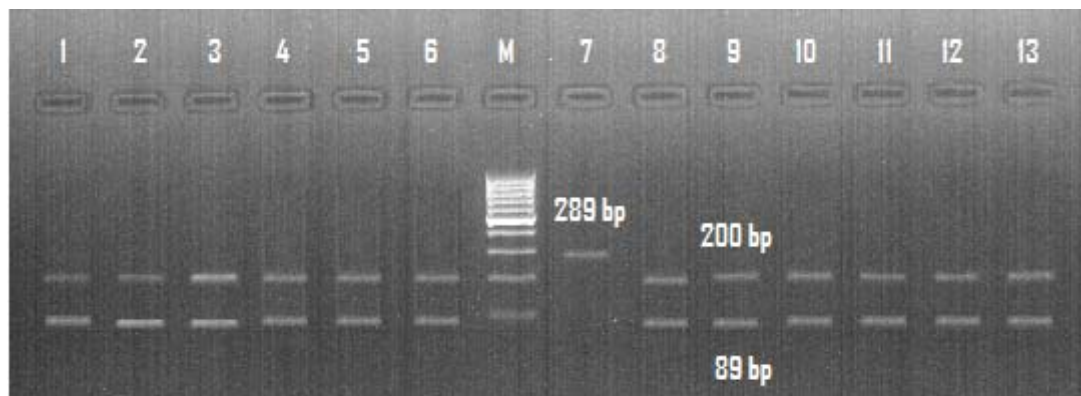


Figure 7. PCR-RFLP of primer I of the leptin gene on 2.5% agarose gel using *HphI* RE in Murrah buffaloes.

Lane 1-6 and 8-13 : 2 Bands (200 bp and 89 bp)

Lane 7 : PCR Product (289 bp)

Lane M : 100 bp Molecular Marker

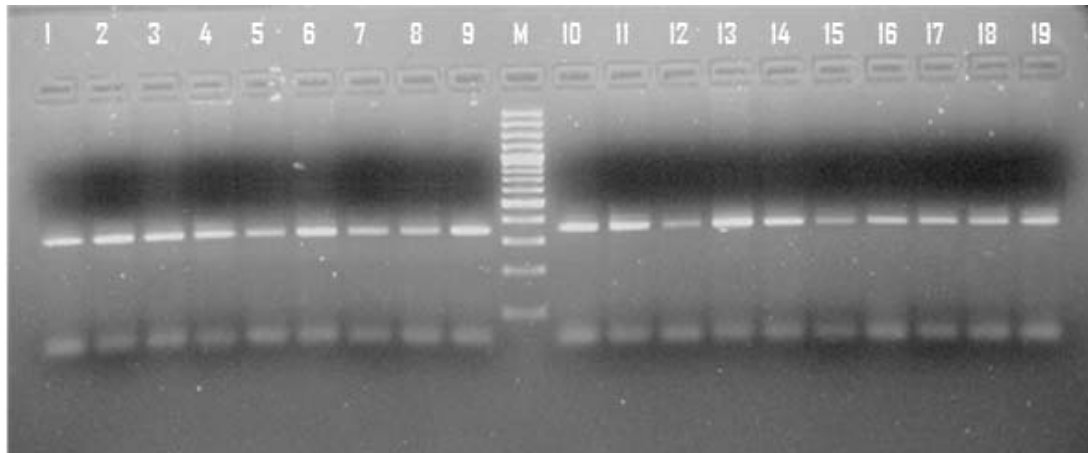


Figure 8. PCR-RFLP of primer II of the leptin gene on 2.5% agarose gel using *AluI* RE in Murrah buffaloes.

Lane 1-9 and 10-19 : 2 Bands (55 bp and 350 bp)

Lane M : 100 bp Molecular Marker

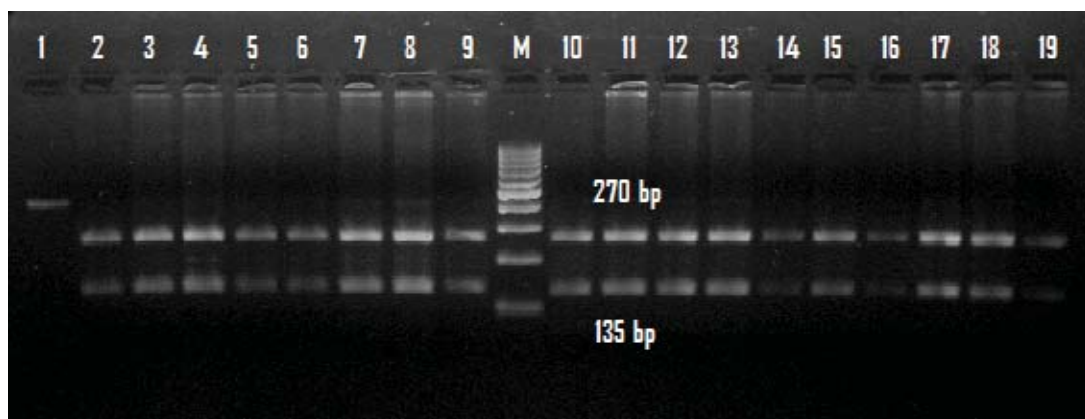


Figure 9. PCR-RFLP of primer II of Leptin gene on 2.5% agarose gel using *AciI* RE in Murrah buffaloes.

Lane 2-9 and 10-19 : 2 Bands (135 bp and 270 bp)

Lane 1 : PCR Product (405 bp)

Lane M : 100 bp Molecular Marker

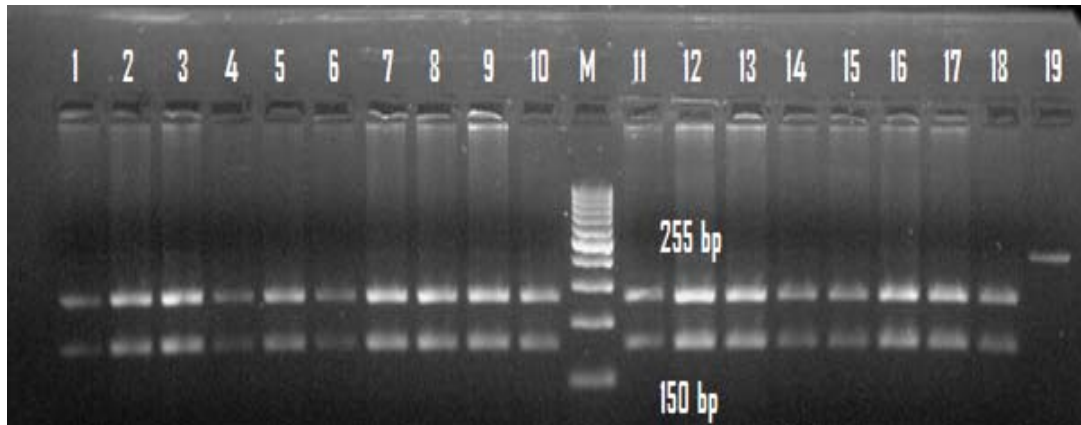


Figure 10. PCR-RFLP of primer II of the leptin gene on 2.5% agarose gel using *MspI* RE in Murrah buffaloes.

Lane 1-10 and 11-18 : 2 Bands (150 bp and 255 bp)

Lane 19 : PCR Product (405 bp)

Lane M : 100 bp Molecular Marker

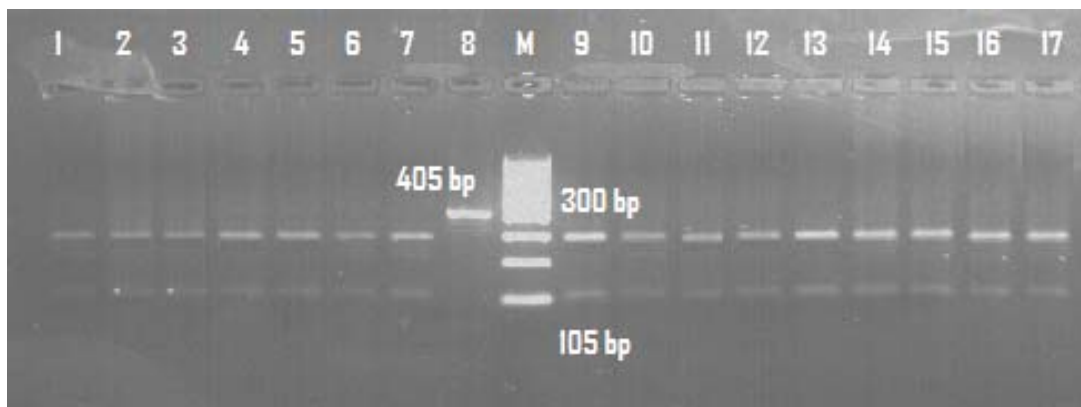


Figure 11. PCR-RFLP of primer II of Leptin gene on 2.5% agarose gel using *Sau3AI* RE in Murrah buffaloes.

Lane 1-7 and 9-17 : 2 Bands (300 bp and 105 bp)

Lane 8 : PCR Product (405 bp)

Lane M : 100 bp Molecular Marker

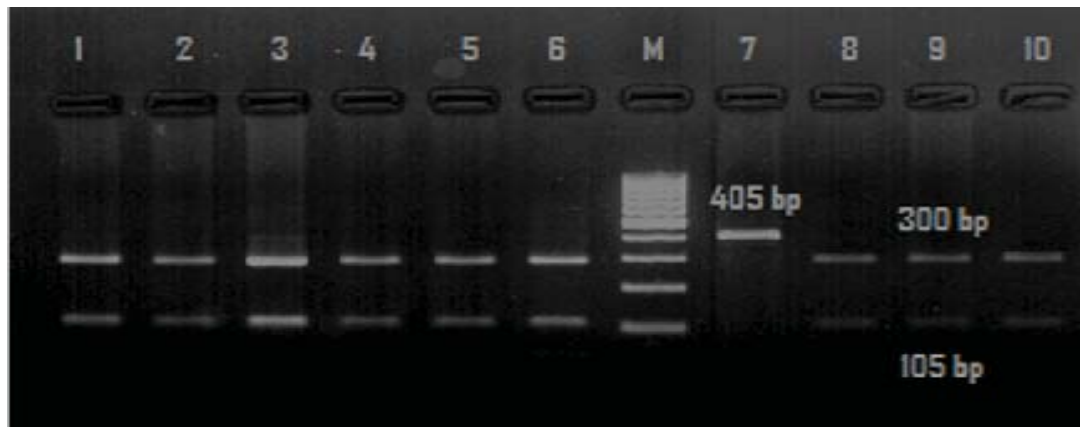


Figure 12. PCR-RFLP of primer II of the leptin gene on 2.5% agarose gel using *HphI* RE in Murrah buffaloes.

Lane 1-7 and 9-10 : 2 Bands (300 bp and 105 bp)

Lane 8 : PCR Product (405 bp)

Lane M : 100 bp Molecular Marker

GGATTAGTGCCTTTTCACTTACTGTCATTTCTAGACAATGAATTGCTTTTGAGGAGATGATAGCCATGGCAGACAGC
AAATCTC (A/G) TTGTTATCTGCATCCGAAGACGTGGATGCGGGTGGTAACGGATCACATGGGTGTTCTCTGAGA
TCGGCGACG (G/T) GCCACGTTGGTTTCTTCTGTTTTAGGCCCCAGAAGCCATCCCGGGAAGGAAAATGCGC
TGTGGACCCCTGTA (C/T) CAATTCCTGTGGCTTTGGCCCTATCTGTCTACGTGGAGGCTGTGCCCATCCGCAA
GGTCCAGGATGACACCAAAACCTCATCAAGACGATTGTACCAAGGATCAATGACATCTCACACACGGTAGGGAG
GGACTGGGAGATGAGGTAGAACTGTGGCCATCCCTGGGGGAACCCCAAGAGGGC

Figure 13. Clustal W alignment with reference sequence of the leptin gene (Exon-2) in Murrah buffalo depicting SNPs in green.

TGTCACATGGGCACAAGAAGTAAGGGCCCAGGGAGGATGGTGTGGAAGTGGGGAGGAAGAACCTCTATGCTCTAG
GGAAAGGCAGAGTCAGGGGAGCTCTGAGGAGCTGCCCTCTCTCCCACTGAGCTCTTGCCTCCCTTTCCTCCTGC
ATAGCAGTCCGTCTCTCCAAACAGAGGGTCACTGGTTTGGACTTCATCCCTGGGCTCCACCCTCTCCTGAGTTT
GTCCAAGATGGACCAGACATTGGCGATCTACCAACAGATCCTCACCAGTCTGCCTTCCAGAAATGTGGTCCAAAT
ATCTAATGACCTGGAGAACCCTCCGGGACCTTCTCCACCTGCTGGCCGCCTCCAAGAGCTGCCCCTTGCCACAGGT
CAGGGCCCTAGAGAGTTTGGAGAGCTTGGGCGTCGTCCTGGAAGCCTCCCTCTACTCCACCGAGGTGGTGGCCCT
GAGCCGGCTGCAGGGGTCACTACAGGACATGTTGCGGCAGCTGGACCTCAGTCCTGGGTGCTGAAGCCTTGAAGG
CCTCTCTTCCCAAAGTCCAGGGAAGAAACCTGAGCTTCTGGCTGTCCACAGGAGA

Figure 14. Clustal W alignment with reference sequence of the leptin gene (Exon-3) in Murrah buffalo depicting SNPs in green.

sequences of the related species available in NCBI Gene Bank viz Bovine, caprine using the NCBI web site Basic Local Alignment Search Tool (BLAST). There exists 97%, 97%, 99%, 98% and 80% sequence homology with *Bos taurus*, *Bos indicus*, *Ovis aries*, *Capra hircus* and *Homo sapiens*, respectively. The higher sequence similarity of buffaloes with sheep and goat than cattle might be due to lower query coverage (47%) in these two species.

CONCLUSION

Bovine leptin gene specific primers amplified the buffalo leptin gene and PCR amplification yielded an amplified product of exon 2 and exon 3 of the bubaline leptin gene. The leptin gene in the Murrah buffaloes included in present study is monomorphic as revealed by PCR-RFLP analysis using *AluI*, *Acil*, *MspI*, *Sau3AI*, *HphI* restriction enzymes. Thus the monomorphic pattern of leptin gene in buffaloes may be a species specific characteristics of buffalo. Eight SNPs were identified while comparing the bubaline leptin gene with the leptin genes of other species. There exists 97%, 97%, 99%, 98% and 80% sequence homology with *Bos taurus*, *Bos indicus*, *Ovis aries*, *Capra hircus* and *Homo sapiens*, respectively.

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ENDOCERVICAL CYTOLOGICAL STUDIES IN ENDOMETRITIS AFFECTED MURRAH GRADED BUFFLOES (*Bubalus bubalis*)

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ABSTRACT

Objective of the study was to develop endometrial cytology as a tool to diagnose endometritis in field situations. A total of 65 cyclic Murrah graded buffaloes consisting of 25 normal healthy in Group 1 and 40 infertile in Group 2 were utilized. For cytological studies at day 0 and 4 of the cycle endocervical secretions by aspiration technique and lavage technique, respectively, were collected. At day 4 of the cycle endometrial biopsies were obtained for histopathological studies.

In the present study, the aspiration at day 0 was successful in 100% attempts but cells were found 92.3% smears. The lavage at day 4 too was successful in 100% attempts and yielded cells in 96.93% smears. At two different sampling intervals both neutrophil count and lymphocyte count differed significantly ($P \leq 0.05$) between Groups 1 and 2. In Group 2, the neutrophil count differed significantly between sampling intervals ($P \leq 0.05$), but not in Group 1. Lymphocyte count did not differ between sampling intervals in both groups, but differed significantly between groups.

In the present study, based on histopathological lesions in Group 2 33, 28 and 39% biopsies were classified into acute, sub-acute and chronic endometritis, respectively. The mean neutrophil and lymphocyte counts did not differ between subgroups. Changes in the endometrium

could be related to cytological findings at day 0 and 4 of the cycle. It was concluded that the cytological studies in genital secretions might conveniently be implemented in field situations.

Keywords: Murrah buffalo, endometritis, endometrial cytology, infertility

INTRODUCTION

Subfertility is a worldwide concern as herd fertility rates have been declining at approximately 1% annually (Royal *et al.*, 2000). In spite of advances endometritis a form of subfertility still remained an economically important cattle fertility problem. One of the problems in tackling endometritis is the lack of a well defined method of diagnosing. Commonly the condition is diagnosed by clinical symptoms, vaginoscopy, rectal palpation, bacteriology and endometrial histopathology. Though the rectal examination is the most preferred method in field conditions, very often it is arbitrary, insensitive, erroneous and inaccurate. The later procedures are laborious and time consuming and required infrastructure and expertise. In the recent past endometrial cytology, which is based on the migration of leucocytes to the site of infections, has been tried elsewhere to rapidly diagnose endometritis. For harvesting

leucocytes from uterine secretions different methods viz. direct swab, cytobrush, aspiration and lavage have been described (Kasimanickam *et al.*, 2005; Barlund *et al.*, 2008). The objective of the present work was to develop endometrial cytology as a tool for diagnosing endometritis in field conditions.

MATERIALS AND METHODS

A total 65 cyclic Murrah graded parous buffaloes consisting of 25 at their first post partum estrus with normal discharge and reproductive organs on rectal examination constituted Group 1 (control) and 40 buffaloes that had the history of infertility ranging from abnormal discharge to repeat breeding were assigned to Group 2. The buffaloes had no history of periparturient complications. The lactation length ranged from 4 months to >1 year. For cytological studies, aspiration technique at day 0 and uterine lavage at day 4 of the cycle were adopted (Kasimanickam *et al.*, 2005; Azawi *et al.*, 2008). The estrus stage of the cycle was diagnosed by symptoms and clinical examination. Estrual discharge was aspirated through a sterile A.I. sheath connected to a 20 ml disposable syringe and was smeared on a clean glass slide. Further, on day 4 of the cycle, 20 ml normal saline solution was infused into uterine lumen through a sterile A.I sheath connected to a 20 ml syringe and allowed to remain there for a few seconds before it was withdrawn by aspiration. The fluid was centrifuged at 1000 rpm for 15 minutes. After discarding the supernatant, cytological smears were prepared from the sediment. Smears were stained with hematoxyllin and eosin stain and examined under oil an immersion lens of a microscope (Singh and Sulochana, 1996).

At day 4 of the cycle, endometrial biopsies

were collected from caudal one-third portion of uterine horns by Albuchin's uterine biopsy catheter. The biopsy samples were placed in 10% neutral buffered formalin, processed by routine procedures and finally stained with haematoxyllin and eosin stain (Singh and Sulochana, 1996). Statistical analysis of the data was done by adopting computer software programmed for Windows XP (Version 9.0, SPSS Inc. Munich) and Excel (Version 2003, Microsoft).

RESULTS AND DISCUSSION

Cytological Studies

In the present study, the aspiration at day 0 was successful in 100% attempts but cells were found in 92.3% smears. The lavage at day 4 too was successful in 100% attempts and yielded cells in 96.93% smears. In the lavage technique, Kasimanickam *et al.* (2005) failed to obtain samples in 17% attempts, when animals were sampled during the early post partum period. Whereas Gilbert *et al.* (2005) and Barlund *et al.* (2008) did not report failure of sampling with the lavage technique in early postpartum cows.

Neutrophils. The neutrophil count in cytological smears obtained at day 0 of the cycle was 4.60 ± 0.64 and $45.69 \pm 3.88\%$ and at day 4 of the cycle was 5.6 ± 0.78 and $57.82 \pm 3.50\%$ in Groups 1 and 2, respectively (Table 1). The neutrophil count significantly differed ($P \leq 0.05$) between groups at two different sampling intervals. In Group 1, the neutrophil count did not differ between sampling intervals, while it differed ($P \leq 0.05$) in Group 2. The significant differences between groups at different sampling intervals were in line with Azawi *et al.* (2008). In cyclic Iraqi buffalo, Azawi *et al.* (2008) reported the neutrophil count to be $41.1 \pm 11.91\%$ at

estrus in repeaters, which was significantly higher than $14.0 \pm 2.02\%$ in the normal control group. In the present study, increased neutrophil (Figure 1) count observed in Group 2 might indicate the presence of infection (Azawi *et al.*, 2008).

In Group 2, the elevated neutrophils count observed between sampling intervals could be attributed to the effect of rising progesterone. During the progesterone dominant phase of the cycle, down regulation of the immune function makes the diestrus uterus susceptible to infections (Lewis, 2003; Azawi *et al.*, 2008). Elsewhere, it was stated that the immune suppressive effect of progesterone might have been compensated for by increased neutrophil influx into the uterine lumen (Subandrio *et al.*, 2000). These authors reported neutrophil influx into the luteal phase uterine lumen when cows were challenged with intrauterine immunomodulators. They also recorded a similar phenomenon when ovariectomised cows were challenged with progesterone treatment.

Lymphocytes. The mean lymphocyte count was 1.64 ± 0.47 and $6.46 \pm 1.32\%$ at day 0 and 1.72 ± 0.51 and $6.68 \pm 1.10\%$ at day 4 of the cycle in Groups 1 and 2, respectively (Table 1). At the two sampling intervals, the counts differed ($P \leq 0.05$) significantly between groups. Further, the lymphocyte counts did not differ between sampling intervals. However, in both groups it followed largely a similar pattern between sampling intervals as observed by the positive correlations in Groups 1 ($r=0.428$) and 2 ($r=0.523$).

The perusal of literature on cytological investigations in the genital tract did not yield related publications on lymphocyte populations. Of course, Ahmadi *et al.* (2006) and Yavari *et al.* (2009) reported prevalence of lymphocytes in the early postpartum uterus. Ahmadi *et al.* (2006) reported $1.7 \pm 0.01\%$ lymphocyte count in early

post partum cows. Similarly, Yavari *et al.* (2009) demonstrated lymphocytes $<1\%$ in cervical and up to 4.5% in uterine smears of different degrees of endometritis affected cows.

The uterus is supplied with ample lymphocytic drainage and contains the full range of lymphohaemopoietic cells and molecular regulators required to generate and elicit humoral and cell mediated immunity. Of course, the uterus is exceptional among mucosal tissues, in that ovarian steroid hormones have considerable effect on humoral and cell mediated immunity (Sheldon *et al.*, 2009). The presence of lymphocytes in uterine fluids definitely throws light on the prevalence of uterine infections.

HISTOPATHOLOGY IN RELATION TO CYTOLOGICAL STUDIES

In Group 1, the common endometrial changes characterized by mild to moderate cellular infiltration and slight increase in connective tissue were found. In addition, in a few biopsies, the presence of inspissated material in the glandular lumen and mild periglandular fibrosis without affecting the glandular architecture were noticed and were in line with Moulikrishna *et al.* (2010).

Based on histopathological lesions, in Group 2 biopsies were classified into acute, sub acute and chronic endometritis (Sheldon *et al.*, 2006; Azawi *et al.*, 2008; Chapwanya *et al.*, 2009). In acute endometritis the lesions were characterized by severe congestion, stromal edema, hemorrhages, denudation of luminal epithelium, infiltration of inflammatory cells predominantly neutrophils and few lymphocytes in the stratum compactum and stratum spongiosum.

In subacute endometritis, commonly the

Table 1. Cytological observations in endocervical smears at Day 0 and 4 of the cycle (Mean \pm SE).

Group	Neutrophils (%)			Lymphocytes (%)		
	Day 0	Day 4	't' Value	Day 0	Day 4	't' Value
1	4.6 \pm 0.64 (25)	5.6 \pm 0.78 (25)	0.99 ^{NS}	1.64 \pm 0.47 (25)	1.72 \pm 0.51 (25)	0.11 ^{NS}
2	45.69 \pm 3.88 (35)	57.82 \pm 3.5 (38)	2.32 *	6.46 \pm 1.32 (35)	6.68 \pm 1.1 (38)	1.13 ^{NS}
't' value	10.45*	14.54*		3.43 *	4.08 *	

Figures in parenthesis indicate the number of observations.

*P \leq 0.05 NS: Non significant

Table 2. Cytological findings in endometrial impression smears collected from different endometritis sub groups at Day 0 and 4 of the cycle (Mean \pm SE).

Group	Day 0 (%)		Day 4 (%)	
	Neutrophils	Lymphocytes	Neutrophils	Lymphocytes
Acute (15)	30.53 \pm 5.04 ^{NS}	4.13 \pm 1.45 ^{NS}	50.4 \pm 5.88 ^{NS}	5.6 \pm 1.92 ^{NS}
Sub acute (13)	42.85 \pm 7.31 ^{NS}	6.93 \pm 1.57 ^{NS}	54.69 \pm 6.48 ^{NS}	9.07 \pm 1.80 ^{NS}
Chronic (18)	33.72 \pm 7.90 ^{NS}	4.55 \pm 2.21 ^{NS}	42.16 \pm 8.42 ^{NS}	3.56 \pm 1.17 ^{NS}

NS: Non significant

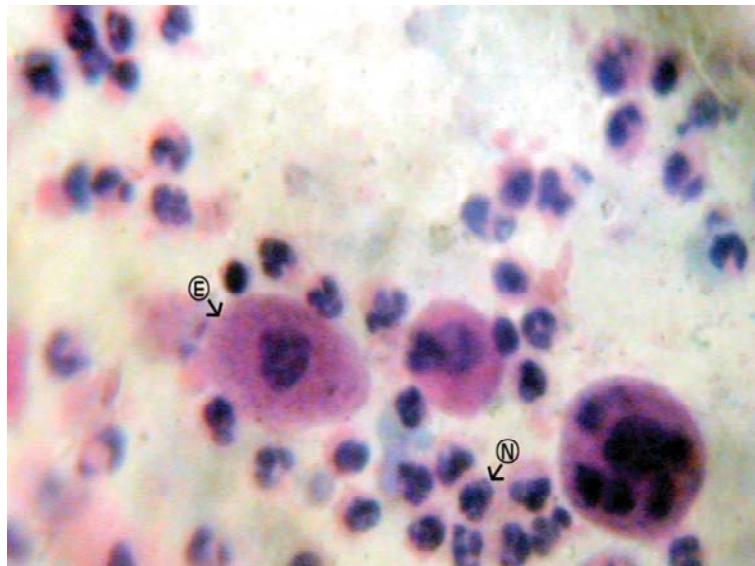


Figure 1. Neutrophils (N) and epithelial cells (E) in endocervical secretions stained with Hematoxylin and eosin (x40).

lesions were characterized by extensive denudation of the luminal epithelium, stromal edema and mild infiltrations of neutrophils, lymphocytes and few macrophages in the stratum compactum and stratum spongiosum, vascular hyalinisation, and peri vascular edema were found. Proliferation of fibroblasts, histiocytes and lymphocytes in the sub epithelial zone and nesting of glands in a few sections, mild periglandular fibrosis and focal lymphoid aggregates were observed. Periglandular fibrosis characterized by 2-3 concentric layers of spindle shaped fibroblasts around uterine glands was recorded.

In chronic endometritis, the lesions were characterized by extensive desquamation of luminal epithelium, severe infiltration by lymphocytes, plasma cells and macrophages, connective tissue proliferation and vascular hyalinization were observed. Fifty percent of the biopsies revealed irreversible degenerative changes characterized by glandular atrophy, pyknotic nuclei in the glandular epithelium, and perivascular and periglandular fibrosis and cystic glands with inspissated material in the glandular lumen were recorded.

In Group 2 33, 28 and 39% of the biopsies were found to have acute, sub acute and chronic inflammatory changes, respectively. Further, the mean neutrophil count was 30.53 ± 5.04 , 42.85 ± 7.31 and $33.72 \pm 7.9\%$ at day 0 and 50.4 ± 5.88 , 54.69 ± 6.48 and $42.16 \pm 8.42\%$ at day 4, in acute, sub acute and chronic endometritis, respectively (Table 2). Neutrophil counts did not differ between sub groups of endometritis. This implied that neutrophil influx into luminal contents was by and large found to be similar in sub groups of endometritis.

In case of lymphocytes too, the mean counts at day 0 were 4.13 ± 1.45 , 6.93 ± 1.57 and $4.55 \pm 2.21\%$ and at day 4 the counts were $5.6 \pm$

1.92 , 9.07 ± 1.8 and $3.56 \pm 1.17\%$ in acute, sub acute and chronic endometritis, respectively, and these did not differ between groups. Of course, the pattern of influx of neutrophils and lymphocytes was in agreement with the established principles of decline as the stage of inflammatory process advances (Cole *et al.*, 1997).

Based on the findings in the present study, it was concluded that cytological investigations in genital secretions might conveniently be implemented even in field situations for drawing appropriate conclusions on the status of uterine health. Further, it was confirmed that the samples obtained from the cervix might provide mirror image of the uterine environment.

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FIELD LEVEL STUDY ON THE BUFFALO BULLOCK: AN EXCELLENT DRAUGHT ANIMAL

Aruna Pal¹ and P.N. Chatterjee²

ABSTRACT

In the dry arid region of Birbhum district of West Bengal, excessive water scarcity, soil structure and texture makes cultivation very much restricted. Fodder production is a luxury here. So it is very hard to maintain high producing stocks. The only viable option is the propagation of draught animals of elite germplasm, which are very hardy and disease resistant to a great extent and can thrive on less feed and fodder, resulting in lowered feeding and maintenance cost. Out of the total of livestock, draught animals comprise about 14.77% in this district, out of which 75.80 % are bullocks (cattle) and 24.19% are buffalo bullocks. The present study was conducted on 810 buffalo bullocks randomly chosen from farmers' doorsteps covering four blocks of Birbhum district of West Bengal. The region lies at the border of Jharkhand state. The average temperature in the summer is 40°C and in winter about 15°C, and the average rainfall is about 1430 mm. Buffalo bullocks were characterized by a heart girth of 72.15 ± 0.43 cm, a body weight of 489.6 ± 7.12 kg, a weight carrying capacity for a pair of bullock of 18.2 ± 0.65 kg, a distance travelled per day of 72.0 ± 0.57 km, a speed of travelling of 9.00 ± 0.04 km/h, a working period during agriculture (approx 4 months in a

year) of 8.0 ± 0.05 h and at other times of 9.5 ± 0.06 h, a working year of 12 months, an average cost of feeding of Rs. 38.5 ± 0.61 per bullock, a working area ploughed of 1805.976 ± 15.483 sq m, and a speed of ploughing of 5.2 ± 0.02 km/h. Buffaloes are very good draft animals with the capability of carrying loads of more than twice those of bullocks. Compared to cattle bullocks, buffalo bullocks can travel longer distances for the whole night around 10-12 h with rest periods of 1-2 h. Buffaloes can work round the year. Although the feed cost is higher than cattle due to more DM intake, buffalo can thrive on more fibrous feed, and their efficiency of energy utilization is far better. However, buffalo bullocks are slow in working efficiency. Buffaloes are observed to be most resistant to diseases, specifically systemic infectious diseases (only 13.39%) compared to other animals, namely goat (36.28), sheep (51.37) and cattle (29.17). One of the most interesting observations is that a negligible number of buffaloes under study had systemic infection in terms of febrile reactions or any type of contagious disease, especially FMD in winter. Since the buffaloes in this area are utilized for draught purposes, the majority of the buffalo bullocks reported wounds (16.96%) caused by ploughs during working, pain in the leg due to mechanical trauma (15.17%)

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and yoke gall (9.82%) due to mechanical injury caused by ploughing and carting. However due to excessive mechanical work, most of the buffaloes reported hypoglycaemia and nutritional (vitamin and mineral) deficiency (15.17%) which is more in summer (17.44%). Buffalo bullocks should be given due recognition considering their load bearing capacity in those remote areas and power of resistance to common diseases. However in national breed upgrading programmes, artificial insemination and selection have been stressed for animals with better milk yield, giving no scope for propagation of draught animals with excellent germ plasm, imparting extreme risk which may result in extinction of this treasure in near future. This possess the scope for immediate conservation of the germ plasm of draught animals as a source of renewable source of energy and select best draught buffalo bullocks by marker assisted selection.

Keywords: buffalo bullock, *Bubalus bubalis*, draught animal, India

INTRODUCTION

Despite motorization on all fronts the use of draught animal power (DAP) is still often more economic than the use of machinery and vehicles, especially in small-scale agriculture and in remote areas. Animals are produced and maintained locally and don't require the infrastructure needed for motorization. Moreover when the value of machines are likely to be depreciated over time, animals may appreciate because of growth. The principal environmental advantage of DAP compared with mechanization is that DAP relies on bio-energy for its creation, maintenance and functioning instead of fossil energy. So DAP provides the renewable

source of energy (FAO, 1994).

The population of draught animals is decreasing at an alarming rate. The reason is that in our national breed upgrading programmes, no emphasis has been given to DAP. However, in some places emphasis has been given to dual purpose breeds. Animal traction is the use of animals [cattle (bulls, oxen and cows), donkeys, mules, horses, camels, water buffaloes, etc], to assist farmers in carrying out their tasks (Simalenga and Joubert, 1997). Draught animal power is used in agriculture for ploughing, harrowing, planting, ridging, weeding, mowing and harvesting; in transport, for pulling carts and loads over a surface, logging and carrying loads (pack animals); in irrigation for driving water-pumps and pulling water from wells; in the building industry, for assisting in earth moving for road works, for carrying bricks, etc and to provide power for the operation of stationary implements such as threshing machines, grain mills and food processing machines.

In West Bengal, the total buffalo population is 764,000. The total buffalo population has decreased by 8.42% in West Bengal, whereas in India, the buffalo population has increased by 1.84% (Livestock Census, 2007). So this is an alarming trend, and conservation needs immediate attention in West Bengal. In the dry arid region of Birbhum district of West Bengal, excessive water scarcity, soil structure and texture makes cultivation very much restricted. Fodder production is a luxury here. So it is very hard to maintain high milk producing animals. The only viable option is the propagation of draught animals of elite germplasm, which are very hardy and disease resistant to a great extent and can thrive on less and coarser feed and fodder resulting in lowered feeding and maintenance cost. Buffaloes are maintained in extensive production systems. Draught animal power being a renewable

source of energy forms the basis for use in the future instead of mechanization or use of motor vehicle.

Thus the present study was undertaken with the objective of phenotypic characterization, draughtability and disease resistance study of buffalo bullocks along with socioeconomic impact on farmers.

MATERIALS AND METHODS

Animals

The present study was conducted on 810 randomly chosen buffalo bullocks from farmers' herds as draught animals from four adjacent blocks (Dubrajpur, Khoyrasole, Rajnagar and Suri-1) of Birbhum district of West Bengal within similar agroclimatic region. The animals were maintained under an extensive system of management on small farms, with a maximum of two buffaloes, on natural grasses and in communal paddocks during the rainy season. Agricultural by-products are used for feeding (basal feed as paddy straw). They are offered a negligible amount of rice gruel and vegetable wastes during the time of heavy load. The animals were maintained by marginal farmers utilizing family labour with minimum investment and reared with simple and traditional technology.

Agro-climatic conditions

The region lies at the border of Jharkhand and has an average temperature in summer of 37°C and in winter of 11.2°C and an average rainfall is about 1430.5 mm (Mishra, 2006). The region is classified as dry-arid, so due to excessive water scarcity, grazing land is very much restricted and fodder cultivation is extremely difficult.

Phenotypic traits

The traits under consideration were heart girth, body weight, height at withers, km travelled

per day, average weight carrying capacity for a pair of bullock, speed of travelling, working period during agriculture (approx 4 months in a year), months of work during the year, average cost of feeding per animal, working area ploughed, distance travelled per day for a pair of bullocks and speed of ploughing. The heart girth and height at withers were measured manually using tape; body weight was estimated from heart girth. Draught traits were obtained from door to door survey of the owners' houses in cooperation with veterinary hospitals and local practitioners.

RESULTS AND DISCUSSION

Animal traction forms the basis of agriculture in these four blocks of Birbhum district. In four blocks, 60.71% of total buffalo were used for draught purposes in terms of ploughing or in cart pulling. In Khoyrasole, Dubrajpur, Rajnagar and Suri-I block, the percentage of total buffalo used for draught purposes were 50.03, 77.6, 38.11, 57.3 percent, respectively (Table 1). In Dubrajpur block, the highest percentage of draught animals was present. Hence draughtability forms the basis of agriculture in Dubrajpur along with other blocks. In these areas, on contrast to 141 tractors and 15 agricultural power tillers, the total number of animal driven ploughs used for ploughing were 33,397 comprising of 9,587 wooden ploughs and 23,810 steel ploughs (Table 1). The highest number of buffalo driven animal carts were present in Dubrajpur block. Hence animal traction forms the major factor controlling the livelihood and economy of the poor people. Animal carts were utilized for the transportation of coal from adjacent coal mines and transportation of agricultural products and by-products from the nearby fields. Photographs of

draught buffaloes were visualized as sick buffaloes in confinement and being examined before treatment (Figure 1, 3 and 4) and buffaloes with bullock cart (Figure 2 and 5).

Draughtability of buffalo bullock

Buffalo bullock were observed to travel longer distance for the whole night around 10-12 h with rest periods of 1-2 h (Table 1). Buffalo bullocks (18.2 ± 0.65 kg) were observed to be very good draft animals with the capability of carrying loads of more than twice those of cattle bullocks (8.38 ± 0.19 kg) (Table 1). Buffalo bullocks were observed to travel at much higher speeds (9.0 ± 0.04 km/h) than cattle bullocks (Table 1).

This region is based on rain-fed agriculture. Agriculture is practiced once a year for approximately 4 months. During agriculture, buffalo bullocks (8.0 ± 0.05 h) can work for longer periods compared to cattle bullocks (Table 1). During other times, bullocks were utilized for pulling bullock carts. Buffalo bullocks can pull cart for a longer time per day (9.5 ± 0.06 h) compared to cattle bullocks (Table 1). A remarkable characteristic of buffalo bullocks observed was that buffalo bullocks were able to work round the year, whereas the cattle bullocks could work for 6.73 ± 0.16 months during the year.

Other workers have also reported that buffaloes were superior to other draught animals in wet or waterlogged conditions, such as in muddy paddy fields. They can also be used for cart haulage, carrying heavier loads than cattle. In view of the working capacity of buffalo, they have been referred as the "*Living tractor of the east*". In all the rice growing countries of Southeast Asia, the buffalo is used for ploughing mud fields. The large hooves, flexible foot joints, slow but deliberate working attitude is ideal for working in the deep mud of rice fields (Khan and Niamatullah, 2010).

Singh and Barwal (2010) also agreed with the present estimation that buffalo can pull loads more than 6 times of its own body weight, but its usual load carrying capacity is 1-5 to 2.0 tones i.e. 3 to 4 times of its body weight. These loads it can pull for 2-3 h continuously and for 6-8 h in a day during winter and 5-6 h in a day during summer with rest in between.

However, buffalo bullocks are slightly slower in working efficiency compared to cattle bullock, as evident from speed of ploughing (Table 1). However the total work output of buffalo was much superior to cattle. It is evident from Table 1 that working area ploughed per day for buffalo (1805.976 ± 15.483 sq meters) was higher than that of cattle.

Body parameters of buffalo bullocks as draught animals

Buffalo bullocks were found to be much more robust and of higher body weight (489.6 ± 7.12 kg) compared to cattle bullocks (Table 3). The body sizes of buffalo bullock were observed to be much greater (183.26 ± 1.09 cm) than cattle bullock (150.83 ± 1.12 cm) as evident from their heart girth. Average height at withers of the buffalo (133.34 ± 0.95 cm) was observed to be much higher than that of the cattle bullock.

The average body weight of male (Murrah) buffalo was 550 kg and the average height at withers for the male was observed to be 1.42 meters (Singh and Barwal, 2010). Hence it is evident that the buffaloes reared in these areas were not of the Murrah breed of buffalo. It indicates the need for formulating strategy for immediate breed conservation of this excellent draught power.

Economic efficiency of buffalo bullocks

Although feed cost of buffaloes (Rs. 38.5 ± 0.61) was observed to be higher than that of cattle (Rs. 29.95 ± 0.4) due to more DM intake, but

buffaloes can thrive on more fibrous feed and their efficiency of energy utilization is far better.

Another important observation was that most of the buffaloes reared in these regions were castrated male animals. Thus it creates problem in individual selection of buffalo for draught purpose and breeding.

Efficient converter of low quality feed

Buffaloes can utilize less digestible feeds (e.g. rice straw, maize stovers, sugar-cane bagasse etc) better than cattle to thrive. This makes buffaloes easy to maintain using locally available roughage and crop residues. The feeding habits of buffaloes and cattle differ from each other. Buffaloes can consume poor quality pasture or feed and can gain or at least maintain their body weight under long periods of under feeding which may often last over 5-7 months in many Asian countries (Khan and Niamatullah, 2010). The superiority of buffaloes over cattle in digestibility and efficiency of utilization of feed nutrients is manifested only when then two species are fed only low plane of nutrition with course roughages as the main source of energy. Cockrill (1968) and Gilani (1980) reported that the digestibility of wheat straw cellulose was 24.3% for cattle and 30.7% for buffalo.

Enrich soil fertility

Buffaloes improve soil structure and fertility while treading paddy fields. Each year, an adult buffalo produces 4 to 6 tonnes of wet manure plus additional urine as bio-fertilizer to the land. This reduces or eliminates the need for chemical fertilizers as well as provides essential soil humus which chemicals cannot provide.

Secure socio-economic status of farmers

Buffaloes are often used as cash savings and can be sold when needs arise (school fees, marriage, crop failure, debts etc). Thus, these animals ensure socio-economic security for these

marginal farmers.

Disease resistant traits of buffalo bullock

Buffaloes are observed to be most resistant to common diseases (Table 4); specifically systemic infectious diseases (only 13.39%) compared to other species namely goat (36.28%), sheep (51.37%) and cattle (29.17%). Since the buffaloes in this area are utilized for draught purpose, the majority of the buffalo bullocks reported in veterinary hospitals have wounds (16.96%) caused by ploughing or during working, and pains in leg due to mechanical trauma (15.17%) and yoke-gall (9.82%) due to mechanical injury caused by ploughing and carting. However due to excessive mechanical work, buffaloes report hypoglycaemia and nutritional (vitamin-mineral) deficiency (15.17%) which is more severe during summer (17.44%).

Among the diseases, buffaloes suffered mostly from parasitic infestation, particularly in this area. An interesting observation was that none of the buffaloes under the study were affected by lung worm infestation and nasal granuloma (Table 5). Among the buffalo bullocks, digestive or ruminal disturbances in terms of ruminal acidosis, bloat, indigestion, non-specific diarrhoea were much less prevalent (Table 4). When the diseases were classified based on season as in winter and summer, it was observed that none of the animals suffered from ruminal disturbances in winter (Table 6). In addition of the advantage to buffalo of having large size muzzle and high mobility of tongue enable the buffalo to achieve a high rate of intake of forage and crop residue (McDowell *et al.*, 1995). Further, greater weight of rumen in buffalo harbours a larger microbial population indicating better conversion of forage. Rumen asidosis is seldom observed in buffaloes due to their high rate of saliva secretion and thus maintaining rumen pH in a better way.

None of the buffaloes suffered from ectoparasites in winter (Table 6). One of the most interesting observations is that, negligible number of buffaloes under study had systemic infection in terms of fever or any type of contagious disease, especially FMD in winter (Table 6). Localized eye and ear infestation was found to be highest in buffaloes (Figure 4). The reason for this may be that buffaloes have the practice of wallowing in water. Localized ear infection may result due to penetration of infected water into the ear.

Nutritional deficiency, especially in terms of vitamin A deficiency and phosphorus deficiency, was observed to be very common in these areas. Around 15.17% of the draught buffaloes were reported to suffer from vitamin-mineral deficiency (Table 4). Because the area is extremely dry, fodder cultivation is very much restricted in these areas. The animals' diets become deficient in vitamin A due to lack of green fodder. Samples from local feed and soil revealed deficiency in phosphorus. The animals become weak and debilitated. Supplementation with vitamin-mineral mixture improved the condition. This demands special emphasis on supplementation of area specific mineral mixture fortified with vitamins.

Similar reports were available regarding disease resistance of buffalo. Lameness and pink eye (due to vitamin A deficiency) were unknown in buffalo (Annon, 2012). In earlier observations (Pal and Chatterjee, 2010a) the disease resistance of buffaloes was already established.

Future scope for selection of buffalo as draught animals

Since draught buffaloes are castrated males, it is quite impossible to propagate the germplasm of the best draught buffaloes. Hence individual selection is not effective. Sib selection and selection based on pedigree were also not

very practicable since only the female animals can reproduce; males are castrated for the purpose of draughtability. Maintaining females for the purpose of draughtability instead of milk production is quite impracticable and not viable. Experimental evidence has suggested that draught capacity and meat characteristics have a negative correlation with dairy traits (Khan and Niamatullah, 2010).

The only viable option is marker assisted selection (MAS). Like milk production, draughtability seems to be polygenic in inheritance. The growth hormone (GH) gene, the leptin gene and the genes controlling other disease resistant traits like the CD14 gene may act as candidate genes for draughtability. Since the trait of draughtability is one of the most neglected traits so far, the candidate genes for draughtability have not been identified till date. Since draughtability is dependant on body physique and since growth hormone controls this, the GH gene is a promising candidate gene. Studies on the GH gene (Pal and Chatterjee, 2010b) had already established the fact. Other genes coding for body growth as growth hormone receptor (Othman *et al.*, 2012), GH releasing hormone (RajaMurugan *et al.*, 2007), IGF-I (Fatima *et al.*, 2009) etc may also act as candidate genes for draughtability. Research on the CD14 gene, responsible for disease resistance on buffalo has also been conducted (Pal, 2006). Other genes responsible for disease resistance includes defensin (Mossallam *et al.*, 2011), lactoferrin (Kathiravan, 2009) etc may also act as candidate genes. Genes preventing environmental stress like HSP70 gene coding for heat shock protein is also related to draughtability as buffaloes are subjected to less environmental stress, especially heat stress while working. Moreover, due to the dark coat colour of buffaloes, they are subjected to more heat stress. HSP70 protein is well reported to protect

Table 1. An overview of draughtability of buffalo bullocks in four blocks of Birbhum district (As per 17th Livestock Census, 2003).

Blocks of Birbhum district under study	Number of draught buffalo	Total male buffalo	Total buffalo population	No. of Wooden plough	No. of steel plough	Animal cart	Agricultural tractor	Agricultural power tiller
Khoyrasole	2082	2705	4161	2437	7171	5404	18	0
Dubrajpur	4424	4817	5701	1377	9361	9069	71	3
Rajnagar Block	808	1136	2120	2526	5786	3473	33	10
Suri-I	667	791	1164	3247	1492	2754	19	2
Total	7981	9449	13146	9587	23810	20700	141	15

Table 2. Draughtability of buffalo bullocks.

	km travelled per day	Average weight carrying capacity for a pair of bullocks (kg)	Speed of traveling (km/h)	Working period during agriculture (h)	working period other than during agriculture (h)	months of work during the year	working area ploughed per day (sq m)	speed of ploughing (km/h)
Cattle	4.98 ± 0.084	8.38 ± 0.19	5.98 ± 0.06	6.0 ± 0.242	7.01 ± 0.00	6.73 ± 0.16	1569.30 ± 24.85	5.28 ± 0.03
Buffalo	72.0 ± 0.57	18.2 ± 0.65	9.00 ± 0.04	8.0 ± 0.05	9.5 ± 0.06	12	1805.976 ± 15.483	5.2 ± 0.02

Table 3. Phenotypic body parameters of buffalo bullock.

	Heart girth (cm)	Body weight (kg)	Height at Withers (cm)
Cattle	150.83 ± 1.12	298.42 ± 4.66	100.50 ± 1.05
Buffalo	183.26 ± 1.09	489.6 ± 7.12	133.34 ± 0.95

Table 4. Comparative disease incidences of buffalo with other species, Figures indicate percentage of animals suffering from diseases.

SI no.	Disease incidences	Goat	Sheep	Cattle bullock	Buffalo bullock
1	Systemic infection (includes high temperature,...)	36.28	51.37	29.17	13.39
2	Wound (Fresh, maggoted or ulcerated)	6.89	7.33	9.97	16.96
3	Total parasitic infestation	26.09	11.92	41.78	21.41
4	Blood protozoan disease			0.58	
5	Digestive/ruminal disturbances	5.74		6.15	2.67
6	Nutritional deficiency(Vit A & others)	7.96	11.0	7.33	15.17
7	Localized eye/ear infection	2.05	2.75	2.35	4.46

Table 5. Parasitic infestation of buffalo bullocks compared to cattle bullock indicated in percentage.

	Parasitic Worm infestation (Anorexia, diarrhea, colic). Immature Amphistome and Fascioliasis	Lung worm	Hydatid cyst	Nasal granuloma	Humpsore/Legsore (Stephanofilaria)	Ectoparasite
Cattle Bullock	26.39	0.146	1.2	0.439	7.33	6.27
Buffalo Bullock	12.5		0.89		2.67	5.35

Table 6. Season wise classification of diseases.

	Species	Cattle Bullock		Buffalo bullock	
	Season	In Winter*	In Summer**	In Winter*	In Summer**
1	Systemic infection (includes high temperature)	29.5	25.80		17.44
2	Wound (Fresh, maggoted or ulcerated)	13.11	14.74	11.53	18.60
3	Parasitic Worm infestation Immature Amphistome and Fascioliasis (Anorexia, diarrhea, colic).	22.95	17.51	23.07	9.3
4	Lung worm		0.46		
5	Nasal granuloma		1.38		
6	Humpsore/Legsore (<i>Stephanofilaria spp.</i>)	13.93	9.67	3.8	2.32
7	Ectoparasite		1.84		6.97
8	Blood protozoan disease		1.38		
9	Digestive/ruminal disturbances	15.57	2.76		3.48
10	Mechanical trauma (Pain in leg)		10.59	19.23	13.95
11	Yoke gall	0.819	3.68	23.07	5.81
12	Localised ear infection	2.45	2.3	11.53	2.32
13	Nutritional deficiency (vit A & others)	1.63	7.37	7.6	17.44

*Winter (1 st January-15th March and 15th October-31st December)

**Summer (16th March-15th October)



Figure 1. Sick buffaloes in confinement and being examined before treatment.



Figure 2. Buffaloes with bullock cart.



Figure 3. Sick buffaloes in confinement.



Figure 4. Sick buffaloes in confinement and being examined before treatment.



Figure 5. Buffaloes with bullock cart.

cells, tissues, and organs from stress (Kiang and Tsokos, 1998) by promoting the folding of nascent polypeptides and by correcting the misfolding of denatured proteins. Heat shock induced-HSP70 expression has a role in the anti-apoptotic pathway (Sreedhar and Csermely, 2004). The role of HSP70 in buffalo have been established by Madhusudan (2007).

CONCLUSION

Buffalo bullocks may be reckoned as a remarkable draught animal exhibiting disease resistance to the majority of diseases. However little attention has been paid in national breed upgrading programmes (artificial insemination and selection, which have been focused mainly on milch cattle) for propagation of draught animals with excellent germ plasm, imparting extreme risk that may result in the extinction of this treasure in the near future. Here lies the need for immediate conservation of the germ plasm of draught animals as a renewable source of energy. Since castrated male buffaloes are utilized for draught purpose, the scope of conventional selection becomes very much restricted. Marker assisted selection for draughtability needs to be employed after identifying suitable markers.

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EFFECT OF FEEDING DIFFERENTLY PROCESSED SWEET SORGHUM BAGASSE BASED COMPLETE RATIONS ON FEEDING BEHAVIOUR, MILK PRODUCTION AND COST ECONOMICS IN GRADED MURRAH BUFFALOES

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ABSTRACT

The effect of feeding differently processed sweet sorghum (*Sorghum bicolor* (L.) moench) bagasse (SSB) based complete rations on feeding behaviour, milk production and cost economics was studied in 24 lactating graded Murrah buffaloes distributed into four experimental groups of six buffaloes each in a completely randomized design. Experimental complete rations were formulated with SSB and concentrate (50:50) and processed into SSB chopped and concentrate (SSBC), mash (SSBM) and expander extruder pellets (SSBP). The control ration was sorghum straw based complete feed mash (SSM). The eating, rumination and total chewing time (min/d, min/kg DMI and min/kg NDFI) and number of chews for eating, rumination and total chewing (per d, per kg DMI and per kg NDFI) were significantly ($P<0.01$) higher in the buffaloes fed the SSBC ration and significantly ($P<0.01$) lower in buffaloes fed the SSBP ration and comparable among SSBM and SSM rations. The milk yield, 6% fat corrected milk (FCM) yield (kg/d) and total solids, solids not fat (SNF), milk fat and protein yield (g/d) were significantly ($P<0.05$) higher in buffaloes fed SSBP ration and not significant among SSBC, SSBM and SSM rations. The feed conversion ratio (kg/kg milk yield and kg/kg FCM yield) and cost of feed (₹) per kg milk yield and per kg FCM yield was significantly

($P<0.05$) lower in the buffaloes fed the SSBP ration compared to the SSBC, SSBM and SSM rations, while the difference was not significant among SSBC, SSBM and SSM rations. Hence, SSB can be used as an alternative roughage source to sorghum straw economically in ruminant rations.

Keywords: sweet sorghum bagasse, complete rations, buffaloes, feeding behaviour, milk production

INTRODUCTION

Sweet sorghum (*Sorghum bicolor* (L.) moench), a dry land crop, is more water use efficient and has recently been gaining importance as a feedstock for ethanol production (Reddy *et al.*, 2005). In general, it can produce stalk 54 - 69 t/ha (Almodares *et al.*, 2008). The bagasse produced after juice extraction from stalks can be used as animal feed (Jafarinia *et al.*, 2005). The feed value of the sweet sorghum bagasse is not less than the value of non-sweet stem that is currently the mainstay of the feed market in and around Hyderabad, India (Blümmel *et al.*, 2009). Feeding of roughages under complete diet systems improved the palatability and utilization of bulky crop residues (Nagalakshmi *et al.*, 2006). Various processing methods like grinding (Reddy *et al.*,

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1992) and pelleting (Reddy, 1990) improved the DM intake and digestibility of nutrients. Nowadays expanders are being used in the feed industry as an alternative to pelleting for processing livestock feeds (Prasad, 2003 and Nagalakshmi *et al.*, 2006). Even though buffaloes are the efficient utilizers of poor quality roughages, information on the effect of feeding differently processed SSB based complete rations on feeding behaviour and milk production performance of graded buffaloes is not available. Hence, an attempt has been made to evaluate SSB as a sole roughage source in a complete diet processed into varied forms like chopping, grinding into mash and expander-extruder pelleting and study the effect of processing on eating, rumination, milk production and cost economics in lactating graded Murrah buffaloes.

MATERIALS AND METHODS

Twenty-four lactating graded Murrah buffaloes in their above early stage of lactation with an average of 3.0 lactations and weighing about 450 kg were selected from the Dairy Experimental Station, College of Veterinary Science, Rajendranagar, Hyderabad, India and distributed randomly into four experimental groups of six animals each in a completely randomized design (CRD) considering body weight, number of lactations, stage of lactation, daily average milk yield and butter fat content, as uniform as possible at the start of experiment. Experimental complete rations were formulated with SSB and concentrate in a roughage:concentrate ratio of 50:50 (Table 1) and processed into chopped SSB and concentrate (SSBC), SSB based complete diet in mash form (SSBM) and in 16 mm expander-extruder pellet form (SSBP). The control diet was formulated

using sorghum straw (SS) and concentrate in a roughage:concentrate ratio of 50:50 and processed into mash form (SSM) at the feed processing plant, Department of Animal Nutrition, College of Veterinary Science, Rajendranagar, Hyderabad, India. The experimental rations SSM, SSBC, SSBM and SSBP were randomly allotted to the four groups of lactating buffaloes and fed the animals three times per day (d) i.e. about half an hour before milkings at 04.00 and 16.00 h and one time at 10.00 h in-between the milkings for a period of 150 d. Daily feed intake, water intake and milk yields were recorded. Eating and ruminating behaviours were monitored visually for a 24 h period (three shifts of 8 h) during last three d of lactation trial. Eating and ruminating activities were noted every 5 minutes, and each activity was assumed to persist for the entire 5 minutes. To estimate the time spent for eating, ruminating and total chewing per kg dry matter intake (DMI) and neutral detergent fibre intake (NDFI), the actual intake for that d was used. A period of rumination was defined as at least 5 minutes of rumination occurring after at least 5 minutes without rumination activity. Total chewing (TC) time was determined as the sum of total eating and ruminating times. The number of chews per d was calculated by the following formulas developed by Allen (1997). Eating chews (number day) = $-5854 + 84.75 \times \text{eating time (min/d)}$. Ruminating chews (number per d) = $-81 + 71.29 \times \text{ruminating time (min/d)}$. Total chews (number per d) = $-12390 + 80.59 \times \text{total chewing time (min/d)}$.

The milk samples were collected fortnightly during the lactation trial to evaluate quality and quantity of milk constituents. Milk samples were analyzed for fat (ISI, 1961) solids not fat (SNF) (ISI, 1965) methods and protein estimation by 'Turbotherm' and Vapodest' (Gerhard, Germany) analyzer based on the principle of Micro-Kjeldahl

Table 1. Ingredient composition (%) of experimental complete rations.

Ingredient	SSM	SSBC	SSBM	SSBP
Maize	31.0	31.0	31.0	31.0
Ground nut cake	16.5	16.5	16.5	16.5
Sunflower cake	20.0	20.0	20.0	20.0
Deoiled rice bran	23.0	23.0	23.0	23.0
Molasses	5.0	5.0	5.0	5.0
Urea	1.5	1.5	1.5	1.5
Mineral mixture	2.0	2.0	2.0	2.0
Salt	1.0	1.0	1.0	1.0
Sweet sorghum bagasse	-	50.00	50.00	50.00
Jowar straw	50.00	-	-	-

Table 2. Effect of feeding differently processed sweet sorghum bagasse based complete rations on eating and rumination in lactating graded Murrah buffaloes.

Parameter	Ration				Mean±SEM
	SSM	SSBC	SSBM	SSBP	
Eating					
Min/d	204.50 ^b	237.80 ^a	205.00 ^b	177.80 ^c	5.51
Min/kg DMI	14.90 ^b	17.40 ^a	14.83 ^b	12.60 ^c	0.44
Min/Kg NDFI	27.71 ^b	33.06 ^a	26.59 ^b	23.91 ^c	0.86
No. of chews/d	11477.38 ^b	14295.31 ^a	11519.75 ^b	9210.31 ^c	466.99
Chews/kg DMI	836.41 ^b	1046.52 ^a	833.15 ^b	652.70 ^c	36.13
Chews/kg NDFI	1554.95 ^b	1987.98 ^a	1494.17 ^b	1238.99 ^c	69.75
Ruminating					
Min/d	404.80 ^b	478.00 ^a	405.50 ^b	366.00 ^c	10.46
Min/kg DMI	29.50 ^b	44.40 ^a	29.33 ^b	25.94 ^c	2.81
Min/Kg NDFI	54.84 ^b	66.47 ^a	52.60 ^b	49.23 ^c	1.68
No. of chews/d	24573.63 ^b	29795.62 ^a	24627.10 ^b	21811.14 ^c	745.94
Chews/kg DMI	1790.88 ^b	2181.16 ^a	1781.11 ^b	1545.63 ^c	58.94
Chews/kg NDFI	3329.39 ^b	4143.53 ^a	3194.25 ^b	2933.99 ^c	116.85
Total chewing					
Min/d	609.30 ^b	715.80 ^a	610.50 ^b	543.80 ^c	15.93
Min/kg DMI	44.40 ^b	52.40 ^a	44.15 ^b	38.53 ^c	1.28
Min/Kg NDFI	82.54 ^b	99.54 ^a	79.19 ^b	73.14 ^c	2.53
No. of chews/d	36051.00 ^b	44106.82 ^a	36146.85 ^b	31021.45 ^c	1210.92
Chews/kg DMI	2627.29 ^b	3228.77 ^a	2614.26 ^b	2198.33 ^c	94.93
Chews/kg NDFI	4884.34 ^b	6133.73 ^a	4688.42 ^b	4172.98 ^c	186.26

Each value is the average of six observations.

^{abc}values bearing different superscripts in a row differ significantly (P<0.01).

Table 3. Effect of feeding differently processed sweet sorghum bagasse based complete rations on quality and quantity of milk production in lactating graded Murrah buffaloes.

Parameter	SSM	SSBC	SSBM	SSBP	Average ±SEM
Milk yield (kg/d)	5.29 ^b	5.17 ^b	5.54 ^b	6.91 ^a	0.20
6% FCM yield (kg/d)	6.29 ^b	6.24 ^b	6.51 ^b	7.74 ^a	0.20
Total solids (%)	17.73	18.20	18.02	17.62	0.23
Solids not fat (%)	10.38	10.58	10.59	10.50	0.11
Milk fat (%)	7.35	7.61	7.43	7.04	0.16
Milk protein (%)	4.32	4.30	4.38	4.44	0.03
Milk constituents yield (g/d)					
Total solids	937.92 ^b	940.94 ^b	998.31 ^b	1217.54 ^a	4.20
Solids not fat	549.10 ^b	546.99 ^b	546.69 ^b	731.08 ^a	3.45
Milk fat	388.82 ^b	393.44 ^b	411.62 ^b	486.46 ^a	2.43
Milk protein	228.53 ^b	226.45 ^b	242.65 ^b	306.80 ^a	1.80

Each value is the average of six observations.

^{a,b}values bearing different superscripts in a row differ significantly (P<0.05).

Table 4. Effect of feeding differently processed sweet sorghum bagasse based complete rations on feed conversion ratio and cost of milk production in lactating graded Murrah buffaloes.

Parameter	SSM	SSBC	SSBM	SSBP	SEM
Feed intake (kg/d)	12.04 ^a	11.76 ^b	12.13 ^a	12.16 ^a	0.10
Feed conversion ratio (kg/kg milk yield)	2.28 ^b	2.27 ^b	2.19 ^b	1.76 ^a	0.01
Feed conversion ratio (kg/kg FCM)	1.91 ^b	1.88 ^b	1.86 ^b	1.57 ^a	0.12
Cost of feed (₹/d)	96.62 ^a	75.56 ^c	79.15 ^b	81.78 ^b	0.22
Cost of feed/ kg milk (₹)	18.26 ^a	14.61 ^b	14.29 ^b	11.83 ^c	0.07
Cost of feed/ kg FCM (₹)	15.36 ^a	12.11 ^b	12.16 ^b	10.57 ^c	0.06

Each value is the average of six observations.

^{a,b}values bearing different superscripts in a row differ significantly (P<0.05).

method (AOAC, 1997; procedure No. 4.2.02). The costs of the rations were calculated on the basis of processing cost and the prevailing market prices of the feed ingredients. The data was analyzed using the 't' test (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

The eating, rumination and total chewing times (min/d, min/kg DMI and min/kg NDFI) and number of chews for eating, rumination and total chewing (per d, per kg DMI and per kg NDFI) were significantly ($P<0.01$) higher in lactating graded Murrah buffaloes fed the SSBC ration compared to those fed the SSBM, SSBP and SSM rations and significantly ($P<0.01$) lower in buffaloes fed the SSBP ration compared to those fed the SSBC, SSBM and SSM rations and comparable among the SSBM and SSM rations (Table 2).

Higher ($P<0.01$) eating, rumination and total chewing times and greater number of chews for eating, rumination and total chewing in buffaloes fed the SSBC ration might be due the larger particle size and less dense nature of the ration compared to the SSBP, SSBM and SSM rations corroborating the findings of Yang and Beauchemin (2009) in lactating dairy cows fed different chop lengths of alfalfa hay based total mixed rations. Lower eating and chewing time in lactating buffaloes fed the SSBP ration might be due to easier consumption of the pellets (Islam *et al.*, 2000) resulting on higher intakes (McDonald *et al.*, 2002). The rumination time per unit DM intake decreasing with decreasing dietary particle size as reported by Beauchemin *et al.* (2003), Mertens (2000), Yang *et al.* (2001) in cattle corroborates the present findings of lower rumination in the SSBP followed by the SSBM and SSM rations. The level of intake also affects

chewing time as the animals that eat high levels of feed spent less time eating and ruminating per unit of feed (Kovacs *et al.*, 1997).

The milk yield and 6% FCM yield (kg/d) was significantly ($P<0.05$) higher in the buffaloes fed the SSBP ration than those fed the SSBC, SSBM and SSM rations (Table 3). The increased milk production might be due to higher DM intake and efficient digestibility of nutrients in lactating graded Murrah buffaloes fed the SSBP ration than those fed the SSBC, SSBM and SSM rations. The heat treatment during expander-extruder processing might have protected protein from ruminal degradation (Glimp *et al.*, 1967; Broderic, 1975), and it also helps in gelatinization of the starch components of the feed and loosening of the bonds between lignin and soluble carbohydrates (hemicellulose, xylose etc.) which in turn resulted in higher ($P<0.05$) energy digestibility in buffaloes in comparison to those fed conventional diets (Nagalakshmi *et al.*, 2004). Higher ($P<0.01$) milk yield (4% FCM) in lactating graded Murrah buffaloes fed cotton straw based expander-extruder complete pellets over conventional ration (Nagalakshmi *et al.*, 2004) and 5.61 and 7.37 per cent higher 4% FCM and fat yield, respectively in Murrah buffaloes fed maize cob based complete pellets than those fed conventional ration (Reddy *et al.*, 2001a) have been reported. The findings of Khan *et al.* (2010) in cross bred milch cows fed on wheat straw based complete pelleted ration (12.75% higher milk and 14% higher FCM) over those fed on conventional ration also corroborate the present findings.

However, the total solids, solids not fat, milk fat and protein per cent in the buffaloes fed differently processed SSB complete rations and SS complete mash were comparable among all the rations. Significant difference was not

observed in fat and SNF % in Murrah buffaloes fed sugarcane bagasse based expander-extruder pelleted ration over conventional ration (Nagalakshmi and Narasimha Reddy, 2010), and comparable fat % in lactating graded Murrah buffaloes fed maize cob based expander-extruder and conventional ration (Reddy *et al.*, 2001b) have been reported.

The daily average total solids, solids not fat (SNF), milk fat and protein yield (g/d) were significantly ($P<0.05$) higher in buffaloes fed the SSBP ration than in those fed the SSBC, SSBM and SSM rations while, the difference in daily average total solids, solids not fat (SNF), milk fat and protein yield (g/d) was not significant among the SSBC, SSBM and SSM rations (Table 4). This might be due to higher average daily milk yield and 6% FCM yield (kg/d) over the SSBC, SSBM and SSM rations. Higher ($P<0.05$) fat yield in lactating graded Murrah buffaloes fed cotton straw based expander-extruder ration over conventional ration was reported (Nagalakshmi *et al.*, 2004)

The feed conversion ratio (kg/kg milk yield and kg/kg FCM yield) was significantly ($P<0.05$) lower in lactating buffaloes fed the SSBP ration compared to the SSBC, SSBM and SSM rations, while the difference in feed conversion ratio (kg/kg milk yield and kg/kg FCM yield) was not significantly different among SSBC, SSBM and SSM rations (Table 4). This might be due to efficient utilization of nutrients in buffaloes fed the SSBP ration resulting higher milk yield, 6% FCM yield (kg/d) over SSBC, SSBM and SSM rations. A 21.99% lower DM intake/kg FCM production in lactating graded Murrah buffaloes fed cotton straw based expander-extruder complete pellets over conventional ration reported by Nagalakshmi *et al.* (2004) corroborate the present findings.

The cost of feed (₹)[rupees] per kg milk

yield and per kg FCM yield was significantly ($P<0.05$) lower in the SSBP ration compared to the SSBC, SSBM and SSM rations while, cost of feed (₹) per kg milk yield and per kg FCM yield was not significantly different among the SSBC and SSBM complete rations (Table 4). This might be due to higher feed efficiency of the SSBP ration over the SSBC, SSBM and SSM rations. Similarly, higher ($P<0.05$) cost of feed (₹) per kg milk yield and per kg FCM yield in the lactating buffaloes fed the SSM ration might be due to the lower feed efficiency of the ration compared to the SSBP ration and higher cost of sorghum straw (₹ 4) compared to SSB (₹ 1). Lower daily cost of feeding in lactating buffaloes fed a sugarcane bagasse based expander-extruder pelleted ration (₹ 51.43) compared to conventional ration (₹ 73.66) (Nagalakshmi and Narasimha Reddy, 2010) and lower cost of feed/kg 4% FCM production ($P<0.01$) in extruded complete diet (₹ 5.60) compared to conventional ration (₹ 8.42) in lactating graded Murrah buffaloes fed maize cob based complete rations (Reddy *et al.*, 2001b) have been reported.

CONCLUSION

The present study indicated that SSB can be used as an alternative roughage source to sorghum straw economically and feeding of complete rations in the form of expander-extruder pellets proved superior in milk production and lowered the time required for eating and rumination, thereby reducing the energy spent on eating and chewing over chopped and mash forms in graded Murrah buffaloes.

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