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Editor
S. Sophon

Publisher
International Buffalo Information Centre,
Office of University Library,
Kasetsart University

Online available:
http://ibic.lib.ku.ac.th/e-Bulletin
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ABSTRACT

Uterine function is often compromised in buffaloes by bacterial contamination of the uterine lumen after parturition, wallowing and insemination. Pathogenic bacteria frequently persist, causing uterine disease resulting in infertility. The presence of pathogenic bacteria in the uterus causes inflammation, histological lesions of the endometrium, delays uterine involution and perturbs embryo survival. Numerous bacteria in a variety of combinations have been isolated from infected uteri. *Arcanobacterium pyogenes* and gram-negative anaerobes such as *Fusobacterium necrophorum*, as well as, *E. coli*, *Streptococcus spp.*, *Staphylococcus spp.*, and *Pseudomonas spp.* are responsible for toxic puerperal metritis in buffaloes. The incidence rate of uterine infection in buffaloes was much higher than in cattle. The earlier appearance and colonization of *E. coli* and lipopolysaccharide endotoxins in the uterus by affecting the phenotype and function of polymorphonuclear cells, and this might support the co-infection on by *A. pyogenes* and gram-negative anaerobes such as *Fusobacterium necrophorum* at later time.

Keywords: buffalo cow, pathogenesis, bacterial infection, metritis, endometritis, postpartum

INTRODUCTION

Water buffaloes are classified in to two main ‘types’: the river type located in South Asia and the swamp type spread across the South-East Asian region. The Mediterranean buffalo, which some consider to be a third type, is derived from the river type. Postpartum metritis and endometritis are the most important disorders in buffaloes (Azawi, 2006), causing high economic losses due to prolonged days open and prolonged intercalving intervals, resulting in involuntary culling (Taha and Azawi, 2003; Singh et al., 2000). Uterine function is often compromised in buffaloes by bacterial contamination of the uterine lumen after parturition, insemination and wallowing; pathogenic bacteria frequently persist, causing genital diseases, a key cause of infertility (Azawi et al., 2008a). The major problems faced by buffalo breeders and farmers include poor reproductive efficiency and prolonged intercalving intervals (Samad et al., 1984: Oswh-Perera, 1999: Barile, 2005: Perera, 1999: Sah and Nakao, 2006). This can be attributed to factors such as harsh environments (Abdalla, 2003), lack of year-round feed supply and minimal managerial inputs (Perera, 2008), in the majority of farming systems under which buffalo are raised (Sah and Nakao, 2006). In every survey of the factors causing endometritis, metritis and toxic puerperal metritis, dystocia and retained fetal membranes
are identified as of major importance in buffaloes (Singh et al., 2000; Ahmed et al., 2009; Azawi et al., 2007). The presence of pathogenic bacteria in the uterus causes inflammation, histological lesions of the endometrium, delays uterine involution and perturbs embryo survival (Azawi and Taha, 2002; Azawi et al., 2008b). In addition, uterine bacterial infection, bacterial products or the associated inflammation, suppress pituitary LH secretion and perturb postpartum ovarian follicular growth and function, which disrupt ovulation in buffaloes and cattle (Sheldon et al., 2002; Hanafi et al., 2008).

The incidence rate of uterine infection in buffaloes was much higher than in cattle (Usmani et al., 2001; Sheldon et al., 2002; Roman-Ponce et al., 2006; Azawi et al., 2008c; Hanafi et al., 2008). The annual incidences of uterine infections in postpartum cows range from 10 to 50% of dairy cattle (Lewis, 1997), 20 to 75% of the buffaloes (Rao, 1982; Jainudeen, 1986; Usmani et al., 2001). Postpartum metritis is one of the most important disorders in buffaloes (Rao and Sreemannarayana, 1983; Reddy et al., 1986; Singla and Verma, 1994; Singh and Sahni, 1995; Tailor et al., 1997; El-Wishy, 2007). Toxic puerperal metritis (i.e. acute septic metritis) is characterized by increased rectal temperature, depression, anorexia, and a fetid watery vulvar discharge (Azawi et al., 2007). Toxic puerperal metritis can be a severe problem, and uterine infections that are life threatening (Tomnar et al., 1984; Singh et al., 1997; Azawi et al., 2008d). Metritis and endometritis are inflammation of the uterus. Metritis involves the endometrium, the underling glandular tissues and the muscular layer (McEntee, 1990; Lewis, 1997). Endometritis involves only the endometrium, which includes the superficial (luminal) epithelium, the underlying stratus compactum (stromal cells and gland necks) and the stratum spongiosum (gland bodies and stroma) (Azawi and Jajo Azar, 2002), and without systemic signs (Azawi et al., 2008b). These diseases share common etiological factors, predispose to one another and, largely, share common treatment (Azawi, 2006).

In this species, the related knowledge available in the literature is very limited and most studies concerning uterine infection are in cattle. The goal of this review is to present comprehensive current information on the pathogenesis, incidence, bacterial causes, and uterine defense mechanism in buffaloes.

**Classification of uterine infection**

Several systems have been described in attempt to classify and define uterine infection. Uterine infections are generally classified according to clinical signs and degree of severity, which adheres to definitions used by theriogenologists (Noakes et al., 2001). However, frequently the definition or characterization of the various manifestations of uterine disease either lack precision, or definitions vary among research groups and/or were not validated as to their effect on reproductive performance, making assessing the effects of treatment difficult. Often the term endometritis incorrectly includes metritis and endometritis/or is determined solely based on transrectal palpation of an enlarged uterus (Lewis, 1997). During the 15th International Congress on Animal Reproduction (Gilbert, 2004), it was suggested that the research field would be aided by clear definitions of uterine disease that researchers could adopt. Sheldon (Sheldon et al., 2006) provided a clear clinical definition of uterine diseases: toxic puerperal metritis is an acute systemic illness due to infection of the uterus with bacteria, usually within 10 days after parturition. The following clinical signs characterize toxic puerperal metritis.
in buffaloes: a fetid red-brown watery uterine discharge and usually, pyrexia, reduced milk yield, dullness, inappetance or anorexia, and elevated heart rate, and apparent dehydration may also be present (Azawi et al., 2008d). The term metritis is used for animals that are not systemically ill, but have an abnormally enlarged uterus and a purulent uterine discharge detectable in the vagina (Azawi et al., 2008a). Clinical endometritis is characterized by the presence of a purulent (>50% pus) or mucopurulent (approximately 50% pus, 50% mucus) discharge detectable in the vagina after 26 days postpartum [14]. A new technique for the diagnosis of endometritis that has been used recently in bovine gynecology is uterine cytology, mainly to detect subclinical endometritis in clinically healthy cows (Barlund et al., 2008). The proportion of polymorphonuclear neutrophils (PMN) in the total number of endometrial cells is indicative for subclinical endometritis (Westermanna et al., 2010). Different threshold values for the proportion of PMN have been suggested, varying from 5 to 18% (Dubuc et al., 2010). Reports on the use of endometrial cytology for the diagnosis of clinical endometritis, however, polymorphonuclear cells are limited to one recent study that described endometrial cytology as the most reliable method of diagnosing endometritis in cattle (Westermanna et al., 2010). Subclinical endometritis can be defined as endometrial inflammation of the uterus usually determined by cytology in the absence of purulent material in the vagina. A cow with subclinical endometritis is defined by > 18% in uterine cytology samples. Recently Dubuc et al. (2010) defined postpartum endometritis as by its negative effect on subsequent reproductive performance, cytological and clinical diagnostic criteria were taken together to determine the optimal definition of endometritis. They also suggested that clinical endometritis terminology may not be appropriate and that purulent vaginal discharge may be more descriptive. Buffaloes may be classified according to their uterine health status as purulent vaginal discharge only, cytological endometritis only, or both purulent vaginal discharge and cytological endometritis.

Pathogenesis

Following calving the uterus of buffaloes becomes contaminated with bacteria [Azawi, 2006; Azawi et al., 2008a; Azawi et al., 2008e]. Some of these bacteria are harmful and others are not (Azawi et al., 2007; Azawi et al., 2008b). When harmful bacteria are present; the uterus may become infected (Azawi, 2009). One should differentiate between uterine contamination and uterine infection. The uterus of postpartum buffaloes is usually contaminated with a range of bacteria, but this is not consistently associated with clinical disease (Azawi et al., 2008g). Infection implies adherence of pathogenic organisms to the mucosa, colonization or penetration of the epithelium, and/or release of bacterial toxins that lead to establishment of uterine disease (Azawi et al., 2007). The development of uterine disease depends on the immune response of the buffalo, as well as the species and number (load or challenge) of bacteria (Azawi, 2006; Azawi et al., 2008e). The number of pathogenic bacteria in the uterus of postpartum cows may be great enough to overwhelm uterine defense mechanisms and cause life threatening infection (Singh et al., 1986). The postpartum uterus has a disrupted surface epithelium in contact with fluid and tissue debris that can support bacterial growth (Azawi et al., 2008e). The outcome of uterine contamination depends on the number and virulence of the organisms present (Azawi, 2006), as well as the condition
of the uterus and its inherent defense mechanism (Azawi, 2008). A mild to severe endometritis occurs in 90% of postpartum buffaloes during the second through fourth postpartum weeks (Azawi, 2006). Resolution of the inflammation in cattle occurs with time, firstly being restored in the normal cow by 40 to 50 days postpartum (Jainudeen and Hafez, 1993). No information is available on the resolution of postpartum endometritis in buffaloes after normal parturition. The interval from calving to clinically completed involution of the uterus in buffaloes varied widely with a minimum of 25 days (Jainudeen and Hafez, 1993) and a maximum of 74 days (Devanathan et al., 1987; Qureshi et al., 1998). No study is available on the spontaneous clinical resolution of postpartum endometritis in buffaloes. In cattle, approximately three quarters of cows with postpartum endometritis had spontaneous clinical resolution (Gautam et al., 2010). The central question is why buffalo cows have persistent infection after the postpartum period without spontaneous clinical resolution of postpartum endometritis leading to prolonged days open and prolonged intercalving intervals. This could be due to the prolonged interval from calving to clinically completed involution of the uterus in dairy buffaloes (Qureshi et al., 1998) and to the period of postpartum anestrous or anestrous, which is usually longer in buffalo than in cattle (Dobson and Kamonpatana, 1986; Devanathan et al., 1987).

Further studies in postpartum buffaloes concern the release of acute phase proteins after parturition that helps to promote tissue repair. Following the inflammatory process, hydroxyproline or prostaglandin (PG) F2α metabolites are released to enhance neutrophil chemotaxis and the ability of neutrophils to ingest bacteria and plasminogen activators. They are specific serine proteases that convert plasminogen to plasmin and are likely to play an important role during the inflammatory process of the uterus. Further study of them is needed to understand the impairment of spontaneous clinical resolution of postpartum endometritis.

A variety of species of bacteria, both gram-positive and gram-negative aerobes and anaerobes, can be isolated from the early postpartum uterus (Azawi, 2006; Azawi et al., 2007; Azawi et al., 2008a). Most of these are environmental contaminants. Buffaloes with certain periparturent problems have a reduced ability to control uterine infections. Excess stretching of the uterus, as with hydrops allantois, traumatization of genital tissues during dystocia or obstetric manipulation, predispose for postpartum metritis (Azawi et al., 2007). Metabolic disorders, some traditional practices by farmers and herdsmen in which the hand or implements in are inserted into the vagina of the buffalo cow to stimulate milk letdown, as well as, unhygienic conditions under which animals are allowed to calve, can diminish uterine tonus. In addition, some farmers suture the buffalo cow’s vulva to prevent uterine prolapse immediately after postpartum (Azawi, 2006). Lochia is then retained beyond the normal period, providing a medium for bacterial multiplication (Azawi et al., 2008e). Phagocytosis by uterine leukocytes is reduced in buffalo cow with dystocia, retained fetal membranes and metritis (Azawi et al., 2007). If the uterus is severely debilitated, any of a variety of contaminating organisms can cause a toxic puerperal metritis (Azawi et al., 2008d). In less severe cases, an endometritis is initiated that may become persistent and impair fertility (Usmani et al., 2001; Azawi and Taha, 2002; Roman-Ponce et al., 2006).

**Bacterial causes of uterine infection**

The most common cause of uterine
infection is the pathogenic microorganisms affecting productivity and fertility of buffaloes (Azawi, 2006). Pathogenic organisms isolated from an infected uterus are found generally in livestock environments and are capable of infecting other tissues and organs (Azawi et al., 2008a). Thus, uterine infections are classified as non-specific infections (Sheldon et al., 2004). They are called non-specific infection because the initial colonizing bacterium is not known and the specific bacteria causing the signs of infection are not known (Lewis, 1997). Numerous bacteria in a variety of combinations have been isolated from infected uteri. *Arcanobacterium pyogenes* and *E. coli* are usually associated with uterine infection in buffaloes and cattle (Azawi, 2006; Azawi et al., 2007). The composition of the uterine flora changes somewhat at each recontamination, and no specific combination of organisms is associated consistently with postpartum infections (Azawi et al., 2008e and 2008f). Nevertheless, *Arcanobacterium pyogenes*, either alone or in combination with other bacteria such as the anaerobic *Fusobacterium necrophorum* and *Bacteroides spp* (Azawi et al., 2007), often is associated with uterine infections (Azawi, 2006; Azawi et al., 2007; Azawi et al., 2008f). Intraterine oxygen reductase potential fell in the presence of infection (El-Azab et al., 1988) and mostly the aerobic bacteria, thereby creating an anaerobic environment. This drop in intraterine oxygen reductase potential may be associated with either microorganism metabolism or increased oxygen consumption by polymorphonuclear inflammatory cells. Of the anaerobic microorganisms cultured from cases of uterine infection, *Fusobacterium necrophorum* and *Bacteroides spp.* have been identified (Azawi, 2006; Azawi et al., 2007). When *A. pyogenes* was isolated from uterine fluids, buffaloes developed severe endometritis and usually were infertile at first service (Usmani et al., 2001; Roman-Ponce et al., 2006). Azawi et al. (2007) suggested that organisms other than *A. pyogenes* and gram-negative anaerobes such as *Fusobacterium necrophorum*, as well as, *E. coli*, *Streptococcus spp., Staphylococcus spp.,* and *Pseudomonas spp.* are responsible for toxic puerperal metritis. The growth of anaerobic bacteria may enhance the establishment of *A. pyogenes* and lead to the development of severe uterine infections. Indeed, *Fusobacterium necrophorum* produce leukotoxin (Baron, 2004; Carter, 2004), while *Bacteroides* produce substances that prevent bacterial phagocytosis and *A. pyogenes* produce a growth factor for *Fusobacterium necrophorum* (Azawi, 2008). *Bacteroides* and *Fusobacterium* species are prevalent in the indigenous flora on all mucosal surfaces. Tissue necrosis and poor blood supply lower the oxidation-reduction potential, thus favoring the growth of anaerobes (Baron, 2004). In addition, *Fusobacterium necrophorum* is frequently a secondary invader and mixed infection with *A. pyogenes* is not common (Azawi, 2008). In addition *F. necrophorum* produces a variety of extra-cellular products including hemolysin, hemagglutinin, adhesions, platelet aggregation factor, proteases and DNase .The significance of these products relative to virulence is not clear (Carter, 2004). Azawi et al. (2007) suggested that the earlier appearance of *E. coli* in the uterus affected the phenotype and function of polymorphonuclear cells, and this might support the co-infection on by *A. pyogenes* at a later time.

**Uterine defense mechanisms**

Anatomical and functional barriers mediate effective defense against reproductive tract invasion by environmental organisms as well as nonspecific and specific immune responses.
Dhaliwal et al. (2001) stated that the uterine defense mechanisms against contaminant microorganisms were maintained in several ways: anatomically, by the simple or pseudostratified columnar epithelium covering the endometrium; chemically by mucus secretions from the endometrial glands; immunologically, through the action of polymorphonuclear inflammatory cells and humoral antibodies, but the degree of interaction is not clear. Disruptions of these mechanisms allow opportunist pathogens, mostly microorganisms found in the posterior gastro-intestinal tract and around the perineal area (Azawi et al., 2008e), to colonize the endometrium and cause an endometritis (Azawi, 2008; Sheldon et al., 2008). A degree of bacterial contamination of the uterus usually occurs during, or immediately after, parturition (Azawi, 2006; Azawi et al., 2007; Azawi, 2008). Bacterial contamination of the uterus may also occur during coitus or insemination (Taha and Azawi, 2003; Azawi, 2008). Also in buffaloes, bacterial contamination of the vagina and other external reproductive organs might occur during wallowing (Jainudeen, 1986; Azawi, 2006). Whether or not a persistent infection of the uterus becomes established depends upon the level of contamination, the animal’s uterine defense mechanism and the presence of substrates (such as devitalized tissue) for the growth of bacteria (Azawi et al., 2007; Azawi et al., 2008a).

Under normal circumstances, there are several mechanisms, which prevent opportunist pathogens from colonizing the genital tract. The major anatomical barriers between the contaminated world and the relatively sterile environment of the uterus include the vulva, the vestibule (guarded by a muscular sphincter), and the cervix. It should be noted that, although the vulva may appear of little consequences as a barrier, it is, in fact, remarkably efficient at preventing faecal contamination of the tubular genitalia (Sheldon et al., 2008, 2009) as in cattle, while in buffaloes the larger soft loose vulval tissue might reduce its efficacy as a barrier (Azawi, 2006). In cattle and buffaloes, the cervix is formidable barrier composed of series of mucosal lined collagenous rings (Dhaliwal et al., 2001). In addition, the cervical-vaginal mucus (especially the scant, tenacious mucus of the luteal phase) can function as a physical barrier for organisms that would otherwise ascend the reproductive tract (Sheldon et al., 2009). The circular and longitudinal layers of the uterine musculature provide physical propulsion of particular material, including microbes.

Epithelial cells are the first to make contact with potential pathogens that enter the uterus (Wira et al., 2005). Epithelial and stromal cell interactions are critically important for endometrial function, with stromal cells affecting epithelial cells through both the release of soluble factors and the turnover of the extracellular matrix (Wira et al., 2005). Conversely, epithelial cells affect stromal cells function through the release of soluble factors and cell-to-cell contact. Pierro et al. (2001) suggested that PGE2 could regulate epithelial cells proliferation and may be mediated indirectly by uterine stroma.

Estradiol and progesterone have both opposing and complementary effects on the female genital tract with estradiol stimulating epithelization (especially of the vaginal lining and endometrial gland) and vascularization of the endometrium (Sheldon et al., 2009). Progesterone aids in endometrial gland differentiation and enhances uterine gland secretions, reducing cervical mucus production, prevents uterine contractility (Azawi et al., 2008f), and acts as a counter influence to estradiol in immune protective responses of the
reproductive tract (Wira et al., 2005). Cattle are resistant to uterine infections when progesterone concentrations are basal and they are susceptible when progesterone concentrations are increased (Lewis et al., 1997). For example, spontaneous uterine infection in cattle do not usually develop until after formation of the first postpartum corpus luteum although bacterial contamination can be sufficient to induce the onset of puerperal metritis very soon after calving when progesterone concentrations are basal (Lewis et al., 1997; Sheldon et al., 2009). Postpartum cows that received intrauterine infusions of *Arcanobacterium pyogenes* and *E. coli* when progesterone concentrations were basal did not develop uterine infections, whereas all cows developed uterine infections when the bacteria were infused after the onset of luteal function and progesterone concentrations had begun to increase (DelVecchio et al., 1994). In addition, none of the animals that received intrauterine infusions of *Arcanobacterium pyogenes* and *E. coli* during the estrus phase developed uterine infection, but all of those that received *Arcanobacterium pyogenes* and *E. coli* infusions during luteal phase of the estrus cycle developed uterine infections (Dhaliwal et al., 2001; Sheldon et al., 2009). The previous examples clearly support the idea that progesterone converts the uterus from an organ that is resistant to one that is susceptible to infection. In the cycling buffalo cow, the uterus is usually under progesterone influences. That is, the non-pregnant uterus is in the luteal phase (under the influence of progesterone) for about 14 to 15 days of its 21-day cycle (i.e. from about day 3 to 17 after estrus and ovulation) (Perera, 1999; El-Wishy, 2007). It is under its most significant estradiol influence, with no progesterone to counter its effect, for about 1 day (immediately preceding standing estrus). It has been reported that Murrah buffalo have higher overall plasma estradiol concentration than do swamp buffalo and cows. Values at estrus of 31±1.70 pq/Ml (Devanathan et al., 1987) compare with the lower values of 12.9 pq/Ml and 13.0 pq/Ml., for swamp and cows (Glencross and Pope, 1981; Kani et al., 1984; Avenell et al., 1985). The high estradiol concentrations that occur at estrus and parturition cause changes in the number and proportions of circulating white blood cells, with a relative neutrophilia and a “shift to the left” (Azawi, 2008). Moreover, at estrus, the blood supply to the uterus is increased under the influence of estradiol, whilst at parturition there is a massive blood supply to the gravid uterus. This increased blood supply, coupled with the migration of white cells from the circulation to the uterine lumen, enables vigorous and active phagocytosis of bacteria to occur (Sheldon et al., 2009). Estradiol also causes an increase in the quantity and nature of vaginal mucus, which also plays an important role in defense of the uterus against bacteria by providing a protective physical barrier and by flushing and diluting the bacterial contaminants (Sheldon et al., 2009). The immune functions of the uterus were found to be up regulated when estrogens were increased (Dhaliwal et al., 2001). It is difficult to determine whether increased estrogens during follicular phase induced the up-regulation or whether up-regulation was due to the removal of the suppressive effects of progesterone (Dhaliwal et al., 2001). Wira et al. (2005) demonstrated that changes in ovarian estrogens and progesterone regulate uterine immune function. The effect of estrogens and progesterone may seem antagonistic at first, but the two hormones seem to orchestrate uterine immune function in favor of the animal. Indeed, uterine immune function is up-regulated at estrus when there are many opportunities for the introduction of pathogens and down-regulated
during the luteal phase when the uterus is capable of supporting a conceptus, and this down-regulation during the luteal phase seems to allow the uterus to tolerate a fetal allograft (Lewis, 2003). The most critical factor in uterine defense against infection is rapid, physical clearance of inflammatory debris from the uterus after insemination or calving (Azawi, 2006). Compared to cattle, buffaloes have difficulty in clearing this debris from uterine cavity because they have lower estradiol secretion than cattle during estrous phase that decreases the uterine drainage (Kani et al., 1984; Perera, 2011).

CONCLUSION

The incidence rate of uterine infection in buffaloes was much higher than in cattle. The earlier appearance and colonization of E. coli and lipopolysaccharide endotoxins in the uterus by affecting the phenotype and function of polymorphonuclear cells, and this might support the co-infection on by A. pyogenes and gram-negative anaerobes such as Fusobacterium necrophorum at a later time. Serum complement proteins and immunoglobulins in the buffalo genital tract and secretions in the endometrium or other parts of the reproductive tract of the buffalo have not yet been studied as extensively as in cattle. Further studies are needed to understand the uterine defense mechanism in buffaloes and to compare them with those of cattle as most studies concerning uterine defense mechanism have been undertaken in cattle.

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HYDROAMNIOS ASSOCIATED WITH FETAL DEFECTS IN BUFFALOES

Manda Srinivas¹, G. Veeraiah and N. Lakshmi Rani²

ABSTRACT

The present communication reports two cases of defective fetuses associated with hydroamnios in pregnant buffaloes and discusses its management.

Keywords: hydroamnios, renal dysgenesis, cyclopia, gestational accidents, fetal defects, buffaloes

INTRODUCTION

There are many unrelated complications that can occur during gestation in the cow and that may interfere with normal parturition. Hydroamnios is one such condition. It is characterized by gradual filling of the amniotic cavity (Drost, 2007). The condition may be associated with a genetically abnormal or defective fetus (Roberts, 1982). Reports on hydroamnios in buffaloes are scanty; hence, the present communication places on record two cases of hydroamnios associated with fetal defects in buffaloes.

CASE HISTORY AND CLINICAL OBSERVATIONS

Case I: A full-term primiparous buffalo having had dystocia for six hours was attended for obstetrical maneuvering at the farmer’s door step. Its history revealed that the animal had a pear-shaped abdomen that developed slowly as the gestation progressed, and after the rupture of the allanto-chorion, approximately 40 liters of thick viscid fluid suggestive of excessive accumulation of amniotic fluid had escaped from the birth canal. On clinical examination the animal was dull and depressed. Detailed obstetrical examination revealed a fully formed defective fetus in posterior longitudinal presentation, dorso-sacral position and ankylosed hind limbs extending into the birth canal.

Under posterior epidural anesthesia with proper lubrication, the defective fetus was delivered manually by moderate traction. Upon delivery the abnormal fetus revealed ankylosed left knee, right forelimb fetlock, left hock and right hind limb fetlock with anterior deviation. The fetus also had a partially ankylosed defective lower jaw (Figure 1). Autopsy of the fetus revealed small firm kidneys suggestive of renal dysgenesis. The placenta was

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Figure 1. Defective fetus with ankylosed jaw and limbs.

Figure 2. Defective fetus with deformed face (Cyclopia) and ankylosed limbs.
expelled normally within one hour after manual delivery of the defective fetus.

**Case II:** A pluriparous buffalo at the seventh month of gestation was presented to the dispensary with a history of a pear-shaped abdominal enlargement not correlating with the gestational age and straining since one hour after rupture of allanto-chorion with an impending abortion. Detailed obstetrical examination under posterior epidural anesthesia revealed an intact amniotic sac, its rupture lead to escape of about 50 liters of thick viscid amniotic fluid. A defective fetus was palpable deep in the uterus and was delivered per vaginum by mild traction. The defective fetus had a central median eye with two eye balls (cyclopia), a poorly developed rostrum, a lower jaw longer compared to the upper, a deformed mouth, and a protruding tongue with ankylosed limbs (Figure 2). The animal had retention of fetal membranes, which were allowed to shed naturally at 96 h post delivery. Secondary bacterial invasion was prevented by administering Inj. Enrofloxacin 20 ml IM for 5 days along with supportive therapy (Hugo Eiler, 1997).

**TREATMENT AND DISCUSSION**

Supportive therapy for these cases included isotonic salines IV at delivery to counteract stress and dehydration, oxytetracycline 4 gm oblet IU once daily for 3 days, uterine ecbolics for 5 days and Inj lutalyse 25 mg IM immediately after delivery to hasten uterine delivery (Sloss and Duffy, 1980). Both the cases had uneventful recovery with good subsequent fertility. The findings recorded in the present report are in agreement with those of Drost (2007) who reported that the uterus contains viscid fluid which may contain muconium at parturition with a defective fetus. Hydroamnios can be due to hereditary or fetal anomalies with impaired deglutination or renal dysgenesis or agenesis that leads gradual accumulation of amniotic fluid (Roberts, 1982; Drost, 2006). Hereditary causes in the present report can be ruled out as these cases are sporadic with different sires. Defective fetuses with renal dysgenesis in Case I and impaired deglutination due to defective face in Case II might have led to the occurrence of hydroamnios (Roberts, 1982; Drost, 2006). A similar case of dystocia in a buffalo due to fetal monster accompanying hydrops amnii was reported by Sathya et al. (2006) who opined that occurrence of this condition in buffaloes is less common and was associated with specific fetal abnormalities of the face. Honparkhe et al. (2010) also have opined that fetal head abnormalities like cleft palate and caltin mark lead to the formation of hydroamions due to impaired deglutination.

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ABSTRACT

The present communication reports an unusual case of dystocia in a buffalo due to a cyclopic calf. The case was successfully managed by caesarean section without any post-operative complications.

**Keywords:** cyclopia, dystocia, buffalo calf

INTRODUCTION

Cyclopia, or cebocephalus, is a congenital anomaly characterized by a single orbit in which the global tissue is either absent or rudimentary; two eye balls are incompletely fused or there is a single median eye (Roberts, 1971). Cyclopia has been recorded in sheep (Bryden et al., 1971), goat (Sivasudharsan et al., 2010), cows (Gupta and Anand, 2002; Ozcan et al., 2006). However, this anomaly has been rarely reported in buffaloes (Thippeswamy et al., 1996). This paper puts on record of a rare case of dystocia in a Murrah buffalo, caused by a fetus with an atypical form of cyclopia.

HISTORY AND OBSERVATIONS

A primiparous Murrah buffalo was presented with the history of full-term pregnancy with the complaint of dystocia for the previous 10 h. Pervaginal examination revealed complete dilatation of cervix along with a foetus in anterior presentation with an enlarged head firmly stuck in the birth canal. Further attempts to relieve dystocia by repulsion and traction failed and thus, decision was taken to remove the foetus by caesarian section through lower flank laparohysterotomy under local infiltration analgesia with 2% lignocaine. Post-operatively, the animal was given 5 litres of 5% dextrose saline intravenously, enrofloxacain 20 ml intramuscularly for five days and meloxicam 15ml intramuscularly for three days.

RESULTS AND DISCUSSION

A normal-sized male calf was delivered with the presence of a single orbit in the middle of a spherically enlarged head. Examination of the orbital cavity revealed that eye globe was replaced by fatty tissue surrounded by rudimentary eyelids. The nasal region and anterior nares appeared

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to be poorly developed with a single opening. Upper lip appeared like a thin fold of skin with complete absence of muzzle. The upper jaw was short and did not extend to the level of the lower jaw (Brachygnathia superior) along with absence of dental pad and incisor teeth. A deep fissure in the hard palate communicating with nasal cavity was observed on examination of oral cavity. The ears were absent and the external meatus were completely blind (Figure 1).

An abnormal foetus arises when a threshold of genetic and environmental insults is reached and the fetal compensatory mechanisms are overwhelmed. The lack of uniform chromosomal findings in various human cyclopic subjects suggest that factors other than cytogenetic imbalance like gene mutation and environmental agents may be of primary etiologic importance (Cohen, 1966). The single median eye is the result of fusion of two optic grooves in the midline of two eye forming units (Adelmann, 1929) due to defective development of ventral diencephalon (Garzozi et al., 1985). Binns et al. (1963) studied this malformation in newborn lambs in a flock of sheep and stated that this anomaly arises due to ingestion of *Veratrum californicum* in pregnant ewes. According to Roberts (1971) the period of embryo and embryogenesis in bovines is the 12th to 45th day of gestation and this duration represents the most susceptible period for teratological insult. In the present case, the dam might have been exposed to teratogens during the early gestation leading to the cyclopic condition in the calf.

REFERENCES


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*S*Continued from page 14


ABSTRACT

Tetanus is a fairly common disease occurring in all types of livestock. It is relatively rare in buffalo, but due to unhygienic practice following parturition, the umbilicus or the navel easily gets infected through contamination of soil or faeces and buffalo calves can get tetanus. A case report of neonatal tetanus in buffalo with typical clinical signs is described in the present study.

Keywords: buffaloes, Bubalus bubalis, calf, neonatal, tetanus

INTRODUCTION

Tetanus is a non contagious, non febrile infectious disease of mammals caused by exotoxin tetanospasmin produced by the vegetative stage of Clostridium tetani under anaerobic conditions. Tetanus occurs in all parts of the world and is most common in closely settled areas under intensive cultivation. It occurs in farm animals as individual or sporadic cases but outbreaks are common following wounding management procedures. Although recovery rate is high in adult cattle, the case fatality rate may be as high as 80% in young ruminants (Radostitt et al., 2000). Apart from other factors, one important reason for neonatal tetanus is infection in the umbilical cord due to unsanitary conditions at parturition (Suleman, 1982). A case of this type in a male buffalo calf is reported herewith.

CASE HISTORY, CLINICAL SIGNS AND DIAGNOSIS

A one-month-old male buffalo calf was presented to the clinics of the faculty with a history of rigidity of the entire body, hyperesthesia, inability to suckle and stand and also no defecation and urination for the previous two days. Upon clinical investigation, the calf displayed anxious and alert expressions. The ears were erect. Hyperesthesia was evident. Saliva was drooling from mouth. There was great stiffness in all the limbs. The calf was unable to stand, if forced, it adopted a “sawhorse” or “wooden horse” posture (Figure 1). It had great difficulty in walking and was prone to fall. Even after the calf fell, the limbs remained in the state of tetany. The hind limb stuck out stiffly behind and the fore legs forward (Figure 2). Opisthotonus was marked. There were occasional tremors of muscles of the face and limbs.

The jaw was completely locked and difficult to open. However the temperature (102°F) and pulse (72/minute) were within normal limits. Also there was no prolapse of third eyelid and no stiffness in...
Figure 1. “Sawhorse” or “wooden horse” posture.

Figure 2. The hind limb stuck out stiffly behind and the fore legs forward.
the tail. The umbilicus was not properly healed and was found soiled with faeces. Upon detailed anamnesis and clinical investigation the case was tentatively diagnosed as neonatal tetanus.

**TREATMENT, RESULTS AND DISCUSSION**

Soon after diagnosis, the treatment was initiated as per the principles given by Radostit *et al.*, 2000, i.e. elimination of causative bacteria, neutralization of residual toxin, control of muscle spasm and maintenance of hydration and nutrition. The calf was injected with AC vet (product of Intas Pharmaceuticals, India) 1 gm intravenously (i/v). Injection multivitamin 10 ml slow i/v along with 1 litre of injection normal saline solution (NSS). Injection diazepam was given 0.4 mg/kg body weight intra muscularly. Tetanus antitoxin was administered 1000 IU i/v and 500 IU in and around umbilicus after 30 minutes of administration of tetanus antitoxin at umbilicus, the umbilicus was cleaned and debrided aggressively. Thereafter it was irrigated with NSS and sponged with hydrogen peroxide. Despite of all these efforts the calf unfortunately succumbed in a few hours.

Due to unhygienic practice following parturition, the umbilicus or the navel easily gets infected through contamination of soil or faeces during seating. Upon invading in the depth of wound and following average incubation of 10-14 days, the organism under anaerobic condition produces tetanospasmin, which acts upon the muscle and is responsible for the spasm which is a classical sign of the disease (Safarov *et al.*, 1972). Locked jaw, erect ears and constipation are due to the spasm of related muscles. The various clinical signs observed in this case are almost similar to those reported in a cross-bred calf (Bhikane and Kulkarni, 1998). Penicillin being drug of choice helped in the elimination of causative bacteria. Although administration of antitoxin is advocated parentally as well as locally to neutralize the residual effect of toxin (Stauder, 1973), its effect after appearance of clinical signs is questionable. The wound should always cleaned only after 30 minutes of administration of antitoxin locally because aggressive cleaning, debridment or irrigation may facilitate the absorption of toxin. Maintenance of hydration and nutrition is necessary particularly in cases where the animal is unable to eat and drink due to lock jaw. Diazepam was given in order to relive tetany and spasm of muscles. Despite of all these efforts the death in this case may have been due to respiratory arrest/failure following spasm of the muscles of respiration.

**REFERENCES**


COMPARATIVE STUDIES OF THE EFFECT OF BSA VS FCS AS A SUPPLEMENT IN TCM-199 ON THE IN VITRO MATURATION RATE OF BUFFALO OOCYTES COLLECTED FROM SLAUGHTERHOUSE OVARIES

Yosef Deneke1,* , Prem Singh Yadav2, Rajib Deb3 and Trilok Nanda4

ABSTRACT

Availability of developmentally competent buffalo oocytes is critical for in vitro embryo production and application of related biotechniques. The objective of the present study was to assess the effect of BSA in place of FCS as maturation medium supplement on in vitro maturation buffalo oocytes. Oocytes were aspirated from abattoir ovarian follicles of 2-8 mm diameter followed by maturation in TCM-199 supplemented with hCG, PMSG and containing either 0.4% BSA (group-I) or 10% FCS (group-II). Based on cumulus expansion maturation rate, it was assessed that among the two groups, Group 2 showed significantly higher percentage values (89.1±3.5%) as compared to Group 1 (73.9±4.2%).

Keywords: buffalo oocytes, in vitro maturation, BSA, FCS

INTRODUCTION

In buffaloes, embryo transfer has had limited success as compared to other livestock species. The buffalo has low productive capacity, evidenced by a lower number of follicles in the ovary (Agrawal and Tomar, 1998), a high percentage of atretic follicles, changes in acrosomal protein and membrane damage during freezing, because freezing buffalo semen results in acrosomal damage mediated leakage of enzymes, alteration of pH, complete withdrawal of the hydration shell of protein in solution and loss of sperm motility (Nandi et al., 2003).

In vitro maturation (IVM) of unfertilized oocytes has great potential for cattle breeding especially when combined with in vitro fertilization (IVF) and in vitro culture (IVC) and cryopreservation techniques. The culture medium employed for IVM is important in view of its effect on the maturation rate of follicular oocytes and also on embryonic development following IVF (Bavister et al., 1992). The commonly used media are complex, buffered with bicarbonate or HEPES and supplemented with various sera and/or gonadotrophin (FSH/LH) and/or steroid (estradiol 17ß) hormones. It is known that the culture conditions employed for IVM of mammalian oocytes can significantly influence IVF rates and subsequent embryonic development (Brackett et al., 1989; Abdoon et al., 2001). Various types of media viz., TCM-199 (Singh et al., 1989; Totey et al., 1993; Madan et al., 1994; Nandi et al., 2000), Ham’s F-10 (Singh et al., 1989; Totey et al., 1993), MEM (Abdoon et al., 2001) have been

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commonly used for IVM-IVF studies in buffaloes. Among them, TCM-199 is the most commonly used medium. These complex media cannot support oocyte maturation on their own and are usually supplemented with hormones (Totey et al., 1993; Nandi et al., 2000), sera (Bacci et al., 1991; Lu and Hsu, 1991; Madan et al., 1994; Totey et al., 1996) or follicular fluid (Chauhan et al., 1997) which introduce many known and unknown substances to the IVM medium for proper maturation. In the present studies we have evaluated the effect of bovine serum albumin (BSA) and fetal calf serum (FCS) as supplements in TCM-199 as a basic in vitro maturation medium for buffalo oocytes.

**MATERIALS AND METHODS**

**Collection of ovaries**

Buffalo ovaries were collected from a Delhi abattoir in sterile normal saline solution (NSS) supplemented with antibiotics (penicillin 100 IU/ml, streptomycin 50 μg/ml, Hi-Media, India) at 30-35°C in a thermos flask and transported to the laboratory within 4 h of slaughter.

**Retrieval of oocytes**

In the laboratory, the surrounding tissues were trimmed off and the ovaries were washed four to five times with sterile and warm (30-35°C) NSS. The ovaries were then exposed to 70% ethyl alcohol for 2 seconds and finally washed with phosphate buffered saline and immediately blotted with paper towel (Hurtt et al., 2000). Oocytes from ovarian follicles of 2 to 8 mm in diameter were aspirated using 18G needle attached to 5 ml sterile disposable syringe (Dispovan, India) containing 0.5 ml aspiration medium.

The aspiration medium consisted of phosphate buffered saline (Gibco, USA) supplemented with 0.4% fatty acid free embryo tested bovine serum albumin (BSA) (Sigma, USA) and antibiotics. The aspirated suspension was poured into a sterile 90 mm Petri dish (Griener, Germany) having 4 to 6 ml aspiration medium. The dishes were searched under a stereo zoom microscope (Olympus, Japan). Cumulus-oocytes complexes (COCs) were located and picked up from the Petri dish. These oocytes were then washed serially three times with TCM-199 (Gibco, USA), supplemented with 0.4% BSA or 10% fetal calf serum (FCS) (Gibco, Mexico) as per requirement in 35 mm petridish (Griener, Germany) containing 2 ml of the maturation media and finally transferred into washing drops of 100 μl in a 60 mm Petri dish (Grienes, Germany) for a serial wash in 3 drops horizontally. The COCs were graded as described by Nandi et al. (1998):

- **Grade-I**: Compact cumulus oocyte complexes with unexpanded cumulus mass having ≥ 5 layers of cumulus cells and homogenous evenly granular ooplasm.
- **Grade-II**: COCs similar to Grade-I but with 2-4 layers of cumulus cells.
- **Grade-III**: Oocytes with partially denuded or completely devoid of cumulus cells and having an irregular dark ooplasm.
- **Grade-IV**: Oocytes with highly expanded or scattered cumulus cells and an irregular dark ooplasm.

The oocytes of Grades I and II were used for in vitro maturation within 2 h of their removal from the follicles. Oocytes were matured in two groups. Group1 consisted of oocytes matured in TCM-199 supplemented with 0.4% BSA and Group 2 those matured with10% FCS supplementation.
In vitro maturation of oocytes

The maturation media included TCM-199 that was supplemented with either (a) 0.4% BSA or (b) 10% FCS (heat inactivated in water bath at 56°C for 30 minutes) and streptomycin (100 μg/ml, penicillin 100 IU/ml, Sigma, USA), l-glutamine (200 mM, Sigma, USA) and hormones PMSG (10 IU/ml) and hCG (10 IU/ml) (Sigma, USA). After washing twice with 2 ml maturation medium (without hormone supplement) in a 35 mm sterile Petri dish (Griener, Germany), 15-20 COCs were randomly placed in 100 μl of maturation drops (medium + hormone) (Shamsuddin et al., 1993) in a 35 mm sterile Petri dish. The maturation drops were covered with warm (35-37°C) light weight mineral oil (Sigma, USA) and kept for 24 h in a CO₂ incubator at 38.5°C under a condition of 5% CO₂ in air with a relative humidity of 90 to 95%.

Maturation of the oocytes was evaluated after 24 h of culture to access the degree of cumulus cell expansion under a stereo zoom microscope and also the appearance of the polar body using the methods described by Kobayashi et al. (1994) and Nandi et al. (1998).

Degree-0 (slight or no expansion of cumulus cells): oocytes having cumulus cells tightly adherent to the zona pellucida,

Degree-1 (moderate cumulus cell expansion): oocytes having expansion of the cumulus cell mass to 2-diameter away from zona pellucida, cells were homogenously spread and cluster cells were still observed.

Degree-2 (full cumulus cell expansion): oocytes showed enlargement of cumulus cell mass to at least 3-diameters away from the zona pellucida, cells were homogenously spread and clustered cells were no longer present.

RESULTS

A total number of 1954 oocytes were cultured for 24 h in a CO₂ incubator with 5% CO₂ in air under humidified conditions at 38.5°C and their maturation was assessed by the expansion of their cumulus cells and formation of polar bodies (Microphotographs 1, 2, Plate-2). Based on cumulus expansion maturation rate was assessed in two groups which resulted in 819 matured out of 1108 oocytes cultured in TCM-199 supplemented with 0.4% BSA (group-I) and 754 oocytes matured out of 846 cultured in TCM-199 with 10% FCS (group-II), showing 73.9 ± 4.2% and 89.1 ± 3.5 maturation rates in the two, respective groups (Table 1).

DISCUSSION

It has been accepted that the expansion of cumulus cells is important to achieve complete oocyte maturation since it is correlated to the

<table>
<thead>
<tr>
<th>TCM-199 Supplements</th>
<th>No. of oocytes used</th>
<th>No. of oocytes matured</th>
<th>% Maturation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4% BSA</td>
<td>1108</td>
<td>819±6.6</td>
<td>73.9±4.2</td>
</tr>
<tr>
<td>10% FCS</td>
<td>846</td>
<td>754±4.2</td>
<td>89.1±3.5</td>
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</table>

Table 1. Oocytes maturation rates in TCM-199 with supplements 0.4% BSA vs 10% FCS.
fertilization rate and developmental potential in ovine and bovine oocytes (Saigmiller and Moor, 1984; Cox et al., 1993). Based on cumulus-cell expansion, in the present study, about 73.9±4.2% in Group 1 and 89.1±3.5% in Group 2 were considered to be mature after 24 h of in vitro maturation.

In buffalo oocytes, 80 percent maturation has been reported by Totey et al. (1993) in TCM-199 supplemented with FSH, LH and estradiol. Thus, the results in the present study are comparable with those of Totey et al. (1992; 1993a), Singh et al. (1989), Chauhan et al. (1991), Madan et al. (1994 b) and Das et al. (1996b). Brackett et al. (1989) reported 95-100 percent maturation of cow oocytes in the above medium. When the medium is supplemented with other supplements like hormones and follicular fluid, the maturation rate becomes higher: 95-100% (Chauhan et al., 1997). The differences in maturation rates may be due to a number of factors like health of oocytes at the time of collection, physiological and nutritional status of slaughtered animals, time elapsed during transportation of ovaries and composition of the medium. Das et al. (1996b) and Chauhan et al. (1997) did not have desired maturation rates from buffalo oocytes in TCM-199 with FCS and FSH.

Fukuda et al. (1990) reported 74 percent maturation rate for bovine oocytes cultured in TCM-199 supplemented with 10% bovine estrus serum (BES). Totey et al. (1993) also reported a maturation rate of 76% when oocytes were matured in TCM-199 supplemented with hormones and BES.

Cumulus cells are important during oocyte maturation and contribute to the production of cytoplasmic maturation factors (Vanderhyden and Armstorg, 1989) and prevention of hardening of the zona pellucida (De Felici and Siracusa, 1982). Additionally, the cumulus cells secret non-sulfated glycosaminoglycan and hyaluronic acid (Ball et al., 1983), when stimulated by FSH, which causes their dispersion, forming a mucous matrix between and around cumulus cells. Hyaluronic acid promotes the acrosome reaction of epidydimal bovine spermatozoa (Handrow et al., 1982) and the dispersed cumulus cells makes it easier for sperm cells to reach the oocytes, although oocytes without expanded cumulus cells have been fertilized in vitro with high frequency (Schroeder and Eppig, 1994; Sirard et al., 1988).

ACKNOWLEDGEMENT

The first author is thankful to the CCS HAU and to the Director, Central Institute for Research on Buffaloes, Hisar, Haryana, India for providing necessary facilities to conduct the present research work.

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ABSTRACT

Twenty-one dairy buffaloes suffering from clinical mastitis were the subject of this investigation. The affected quarters of the animals were clinically examined. The appearance and consistency of their milk and pH were ascertained and recorded at the sampling site. The pH ranged from 7.00-8.50 (average 7.49). The citrate content of the milk ranged from 24.00-47.50 (average 33.71) mg /100ml. Milk from affected quarters of six buffaloes was cultured; this yielded Staphylococci from four quarters, Streptococci from one, Escherichia coli from two, Bacilli and Klebsiella from one each. On the basis of the lowered citrate content of milk in the affected quarters, the animals were treated with 12 gm or 30 gm of trisodium citrate in 250 ml of water orally once daily till recovery. With 12 gm doses daily, the recovery period was 7-13 days. However, with 30 gm daily, the recovery period was cut-short to 3-5 days. The pH (~6.50) and citrate content of milk (118.30 mg/100 ml) returned to almost normal levels after treatment with trisodium citrate. The bacterial colonies also reduced significantly after the treatment. The constituents of milk, e.g., total protein, fat and lactose, increased significantly after the treatment. The results of our investigation vis-à-vis with other workers have been discussed in detail. It has been proposed logically and conceptually that the initial lesion in the udder is inflicted by the disturbed buffer system of the udder, i.e., lower levels of citrate and free Ca2++ are responsible for this injury in the udder and alkaline pH due to seepage of bicarbonate from blood into the udder providing most conducive conditions for the establishment of environmental non-contagious pathogens. Further, the treatment with trisodium citrate proved safe, economical, very effective, with no discarding of milk, no withdrawal periods and moreover no hazard from residuals in milk and meat.

Keywords: dairy buffaloes, Bubalus bubalis, mastitis, pathobiology, aetiology

INTRODUCTION

Mastitis is a perpetual problem of all milk producing animals (including women). The conservative estimates of economic losses from this malady have been made year after year in almost each and every state world-wide. Several groups of scientists/workers have been engaged globally to find out the exact cause and effective treatment of this most formidable disease. Though much of the work directed towards unveiling the nub of this malady has elucidated intricate biochemical interactions at the molecular level, the solution to
Coming down to that versatile dairy animal, the buffalo (*Bubalis bubalis*), the ‘Asian Black Gold’ having a population of about 130 million globally, suffers extensively from mastitis (Fagiolo and Lai, 2007). Despite the use of best available facilities at hand to understand the pathobiology of mastitis, the problem still remain economically most important to the dairy industry throughout the world. The ideal *modus operandi* to eliminate or reduce the economic losses requires that the definite cause of mastitis be identified and then possible control measures implemented. While scanning the literature on mastitis and biosynthesis of milk in the udder, it became apparent that citrate plays a crucial role in the lactogenesis and maintain udder health through ionic equilibration (Peaker and Linzel 1975; Hyvonen *et al*., 2010). Citrate levels are always low in mastitic milk (Dhillon and Singh 2009. It was hypothesized that replenishment of citrate deficiency with extraneous trisodium citrate might play some protective role against mastitis; hence, these studies were undertaken and the results are communicated in this paper.

**MATERIALS AND METHODS**

Twenty-one buffaloes affected with mastitis were included in this investigation. Milk samples from six buffaloes were cultured and identification made from ensuing colonies. The number of colonies were counted before and after the treatment of the affected buffaloes. Physical examination of the milk and udders was made and the degree of mastitis was graded on the basis of the following scale:

- **+**---- Presence of flakes (See Figure 1)

| ++---- | Serosanguineus milk with admixture of flakes (See Figure 2.) |
| +++- | Curdeled milk with admixture of blood clots (Figure 3.) |
| ++++- | Frank blood with whitish tinge of milk (Figure 4.) |

Grading of milk was compared with the pH of milk from the affected quarters to qualitatively identify the severity of mastitis. Milk citrate content was determined by the method of White and Davies (1963) quantitatively before and after the treatment. The appearance and consistency, the pH and the citrate content of milk were the main criteria in treatment with trisodium citrate.

The treatment consisted of 12 gm or 30 gm of trisodium citrate in 250 ml of water daily as a drench till recovery. No other treatment, such as antibiotic, was given.

**RESULTS AND DISCUSSION**

Table 1 presents the data on the effect of mastitis on various parameters of milk before and after treatment with trisodium citratetrisodium citrate in buffaloes. It was observed that lowered citrate content was restored to normal levels after recovery. There was a relative consistent lowering of udder milk pH of the affected quarters it came down to ~6.50 and the consistency of milk was also restored to normal at recovery, which occurred within 4-7 days after the treatment.

The organisms isolated from different milk samples were: *Staphylococci*, *Streptococci*, *E. coli*, *Bacilli* and *Klebsiella*. The treatment also reduced the number of colonies in the culture at different dilutions of mastitic milk. The treatment with trisodium citrate proved very effective in
Figure 1. Grade +, pH 7.0, Citrate 45.60 mg/100 ml milk.

Figure 2. Grade ++, pH 7.5, Citrate 30.06 mg/100 ml milk.
Figure 3. Grade +++, pH 8.0, Citrate 30.90 mg/100 ml milk.

Figure 4. Tube 1- Grade ++++, pH 8.5, Citrate 24.00 mg/100 ml milk Tube No. 6 containing clear milk after treatment.
controlling clinical mastitis in affected quarters. The treated animals did not show any side effects.

**MOST PROBABLE CAUSE(S) OF MASTITIS**

Thus far, the most common causes of mastitis in dairy animals have been primarily imputed to infectious agents (Zhao and Lacasse 2007). On the basis of infectious causes of mastitis a procession of drugs purported to be effective against these culprits emerged on the scene for controlling ailment in dairy animals. In the beginning these drugs appeared specious. However, continual use of these chemicals proved palliative and presented enormous problem of drug resistance and milk and meat residue dangers for humans (Costa et al., 1997). Moreover, the effectiveness of these antimicrobials in controlling mastitis in dairy animals was rarely more than 50% (Deluyker et al., 2005). Different management practices, e.g. dry-cow therapy, teat dipping, and hygienic measures, were evolved to alleviate effects of this formidable problem, but the devil of mastitis is still rampant and unrelenting. Nevertheless, delving into milk synthesis, the mechanisms of injury to the parenchymatous tissue of the udder appears to be becoming a bit clearer.

It has been widely demonstrated that citrate is the ‘harbinger of lactogenesis’ (Peaker and Linzel, 1975). The same authors further reported that the level of citrate in udder of cow, goat and women shoots up 46 times around parturition. These findings enthuse one to speculate that as citrate plays a pivotal role in milk synthesis, it might possibly be associated with mastitis in dairy animals.

It has been reported extensively that mastitic milk is significantly low in citrate (Oshima and Fuse, 1981; Dhillon and Singh, 2009). Our investigations have also revealed that citrate levels are very low in milk of quarters affected with mastitis (30.90 to 36.53 mg/100 ml). As stated above, a certain minimum concentration of citrate is essential for the normal synthesis of milk in the alveoli in the udder. Therefore, any drop in the citrate content would result in faulty synthesis of milk in a particular quarter(s) of the udder. We have observed that the affected quarters had very low concentrations of citrate as compared with healthy quarters of the same animal (Dhillon et al., 1989, 1991). The deficiency of citrate in a particular quarter may be due to nutritional, metabolic or some other intrinsic unknown factors which need

<table>
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<tr>
<th></th>
<th>Citrate mg/100 ml</th>
<th>pH</th>
<th>No. of Bacterial colonies (dilutions)</th>
<th>Doses of trisodium citrate</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1:10</td>
<td>1:100</td>
</tr>
<tr>
<td>Before</td>
<td>33.71</td>
<td>7.49</td>
<td>111.50</td>
<td>64.33</td>
</tr>
<tr>
<td>(Range) SE</td>
<td>24.00-47.50</td>
<td>7.00-8.50</td>
<td>91-141</td>
<td>47-83</td>
</tr>
<tr>
<td>After</td>
<td>118.30</td>
<td>6.50</td>
<td>90.00</td>
<td>50.83</td>
</tr>
<tr>
<td>(Range) SE</td>
<td>96-146</td>
<td>--</td>
<td>76-102</td>
<td>34-65</td>
</tr>
<tr>
<td></td>
<td>5.82</td>
<td>--</td>
<td>4.89</td>
<td>4.71</td>
</tr>
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</table>
The literature extant on mastitis have clearly revealed that mastitic milk is alkaline (pH 7.0 and above). The normal pH of milk in udder is ~6.50, a level which does not appear to be congenial for the growth of commonly isolated organisms from mastitic milk (Cruickshank et al., 1970). Moreover, the philosophical postulation of invasion by environmental (non-contagious) organisms through the teat canal and establishment of infections in udder seems untenable due to the presence of mechanical, chemical and immunological defense barriers throughout this route (Sordillo and Streicher 2002). Also, it has been demonstrated histologically that 3.1% of samples collected from the udders of slaughtered cows from which microorganisms were isolated did not show any histological changes (Benites et al., 2002). Frost et al. (1980) reported minimal damage to alveolar tissue after ‘moderate’ cases of mastitis induced experimentally with *E. coli*. Furthermore, the notion of infection as a cause of mastitis gets eclipsed by the studies of Newbould and Neave (1965) who could not establish 100% infections in udder through deliberate intra-mammary infusions with *Staphylococcus aureus* cultures. Several cases of clinical mastitis in bovines from which no infectious organism was isolated are on record (Wanasisinghe and Frost, 1979; Bramley et al., 1981; González et al., 1988).

Citrate, indeed, is the main constituent of the buffer system responsible for the maintenance of pH (6.5) in the udder; it regulates the homeostasis between Ca2+ and H+ ions and is the mainstay for the fluidity of milk through its effect on casein micelles (Faulkner and Peaker, 1982; Shennan and Peaker 2000). Citrate in the udder also ensures the sequestration of soluble Ca2+ in milk (Kon and Cowie, 1961) and there is significant synchronization between the two (Holt and Muir, 1979). Hence, deficiency of citrate in the udder would lead to the ‘clumping’ of Ca2+, which manifests as flakes in the mastitic milk. These flakes of Ca2+ probably injure the parenchymatous tissue in the udder alveoli due to reduced moderator effect of citrate. Due to this injury the impermeable barrier to citrate in both directions between blood and milk is disrupted and the inflammatory reaction sets in leading to an array of subsequent events. Such injuries due to free Ca2+ have been reported in myocardium (Fleckenstein et al., 1974; Singal et al., 1979). It has also been recorded that a calcium-dependant endonuclease is associated with necrotic type changes in tissues (Arends et al., 1990). Furthermore, another important ion bicarbonate which transudates from blood into milk during mastitis due to permeability of barrier changes the pH of udder towards alkalinity i.e., 7.0 or more. When such lesions in the udder are created and most conducive environments become available, the udder is subsequently invaded by environmental pathogens culminating in clinical/subclinical ‘infectious mastitis’.

We have also reported that lactose, total proteins and fat are substantially lowered in mastitic milk (Singh et al., 1997; Dhillon et al., 2000; Singh et al., 2007). However, these constituents in milk increased markedly on recovery affected by trisodium citrate therapy. The increment in fat was spectacular (190%) because citrate plays an indirect role through NADPH in *de novo* synthesis of fatty acids in the mammary gland (Garnsworthy et al., 2006). Reduction in the number of bacterial colonies after treatment with trisodium citrate has also been observed by other workers (Dhillon et al., 1995). These observations along with our studies substantiate that this treatment is radical and works at the root cause of mastitis resulting in
remarkable cure of the malady without producing any side effects.

Other contributory factors which further exacerbate the pathogenesis of mastitis are the involvement of neutrophils, infectious agents, plasma proteins, cytokines, free radicals etc., which need exhaustive investigations (Zhao and Lacasse, 2007).

Taking all the findings of the above investigations together, it can be concluded that the initial lesion in the pathogenesis of mastitis is caused by the disturbed homeostasis of citrate and Ca2+ in the udder. On the basis of this hypothesis, we treated the clinical cases of mastitis in buffaloes by administering 12 gm to 30 gm of trisodium citrate in 250 ml of water daily as a drench. Similarly several workers have treated acute and/or sub-acute cases of mastitis in buffaloes with excellent results. They also compared it with other antimicrobials and reported that trisodium citrate was superior as far as the restoration of normal pH and other constituents of milk in the udder was concerned (Yousaf et al., 2010; Prakash et al., 2010).

The treatment of mastitis with this salt has been further standardized by enhanced doses to cut-short the recovery period. The oral dose has been raised to 30 gm in 250 ml of water daily as a drench and the recovery period cut-short to 3-5 days depending upon the severity of mastitis. The disruption of the impermeable barrier between blood and milk in udder, as stated above, formed the basis of intravenous administration of trisodium citrate, which directly reaches the site of injury and normalizes the pH (6.5) in the udder and the infectious agents are scavenged off, thus, restoring ionic equilibrium. The intravenous administration of trisodium citrate in sterilized normal saline as 5% given morning and evening in 50 ml doses and the recovery period shortened to 1-3 days (Singh et al., 2007; Dhillon and Singh, 2009). This treatment was safe, economical, and very effective and avoided culling and discarding of milk with the minimal pain to the animal. Moreover, there were no withdrawal periods or hazards from residual problems in milk and meat.

Based upon our above observations some pharmaceuticals have come up with the formulations containing trisodium citrate as the major content recommended for the treatment or prevention of mastitis in dairy animals and are used extensively with remarkable degree of success in the field.

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ABSTRACT

This study was aimed to investigate protein requirement for maintenance in swamp buffalo calves. Four male swamp buffalo calves (12-18 months of age and 218 kg average body weight) were randomly assigned according to a 4 x 4 Latin square design to receive four levels of crude protein (5, 7, 9 and 11% CP). The experimental consisted of four periods of 21-d, 14 days for adaptation and the last 7 days for sampling feces and urine. Animals were fasted for measuring N-retention on the last 4 days of the experiment. The N requirement for maintenance was estimated by the N balance and N intakes were inserted into a regression equation. The crude protein intake, N excretion in urine and N balance increased (P<0.05) with the increase CP content in the diet. However, dry matter intake and fecal N were not different. The result indicated that the nitrogen requirement for maintenance of growing Thai swamp buffalo calves is 0.75 g N or 4.69 g CP/ kg BW^{0.75}.

Keywords: swamp buffalo, Bubalus bubalis, protein requirement, N balance, protein for maintenance

INTRODUCTION

Nutrition of male buffalo calves is importance as it plays a role in the onset of puberty in calves raised for breeding and it influences the quantity and quality of beef produced by the calves. Study of nutritional requirements of buffalo is necessary as the NRC standard suggested is for dairy or beef cattle. Basra et al. (2003b) reported that lower protein requirements of male Nili-Ravi buffalo calves than cattle calves. Furthermore, research on the requirements of nutrients of Thai swamp buffalo does not exist and adequate information on the nutritional requirements of growing male buffalo calves is lacking. Swamp buffaloes are used for multiple purposes: draft power, transportation, capital, credit, meat, milk, social value, hides, and sources of natural fertilizer for cropping. In many places, buffaloes are preferred over cattle because of their superior quality of milk, better efficiency in utilization of nutrients from poor-quality fibrous tropical feeds and relatively better disease resistance and adaptability to tropical climates (Paul and Patil, 2007). In order to achieve their production potential, buffaloes have to consume their required amounts of nutrient from
their diets. The nutrition of young male buffalo is also important as it plays a major role in the onset of puberty when they are raised for breeding and it influences the quantity and quality of the meat they produced. Dietary protein supply is one of the factors that influence the productivity of animals and is supplied from microbial and dietary sources. Generally, microbial protein supplies 70 to 80% of the required amino acids to ruminants and microbial yield in the rumen depends largely on the availability of carbohydrate and nitrogen (N) in the rumen (Chumpawadee et al., 2006). Feeding high levels of protein may be effective in promoting rapid live-weight gains, especially in growing buffalo (Basra et al., 2003a). Currently, there is insufficient information concerning the effects of protein on nutrient digestibility and nitrogen metabolism in Thai swamp buffaloes. A study of the nutritional requirements of buffaloes is necessary because the current standards of NRC (1996 and 2001) are used for beef or dairy cattle. Although, the nutrition requirements of buffalo have been determined by Kearl (1982), they cannot be accurately applied for swamp buffalo. Basra et al. (2003b) reported lower protein requirements for male Nili-Ravi buffalo calves than cattle calves, and found that the CP requirements for growth may be the same as for Holstein Friesian calves (Basra et al., 2003a). However, an optimum growth rate and feed utilization efficiency, according to inherent genetic potentiality of a particular category of animal, can only be achieved through an accurate valuation of their nutrient requirements (Paul and Patil, 2007). Hence, this experiment was conducted to determine the CP requirements for maintenance of growing Thai swamp buffalo.

MATERIALS AND METHODS

Four male swamp buffalo calves (12-18 months of age and 218 kg average body weight) were randomly assigned according to a 4 x 4 Latin square design to receive four levels of crude protein (5, 7, 9 and 11% CP). The experimental consisted of four periods of 21-d, 14 days for adaptation and last the 7 days for sampling feces and urine. Animals were fasted for measuring N-retention on the last 4 days of experiment. The N requirement for maintenance was estimated by the N balance and N intakes were inserted into a regression equation.

The total feces and urine were collected daily from day 3 to 7 of each collection period. The feces were weighed and mixed well and a 10% sub sample was taken and frozen. At the end of each collection period, the daily fecal samples were bulked for each animal. Ten percent of each mixed bulked sample was taken for chemical analysis and calculations of digestibility of DM, OM, CP, NDF and ADF were done. Urine samples were acidified with 25% H₂SO₄ to keep the final pH of the urine below 3 (to prevent ammonia losses during the day) and then weighed and sampled (similar to feces). Representative samples of feed and feces were collected during the digestibility trial and analyzed according to AOAC (1984) and fiber components (Van Soest et al., 1991). Urine was sampled for determination of urine nitrogen and purine derivative excretion. Urine was diluted five times with distilled water and mixed thoroughly and stored at -20°C for later analysis for N. To estimate the dietary N requirement for growth, the ADG and N intake were inserted into a regression equation: ADG = ADG index x (N intake) - ADG at zero N intake, where the N requirement (Nm) for maintenance equals N intake when ADG is zero.

The data were analyzed by the general linear
models procedure of the Statistical Analysis System Institute SAS (1988) using Duncan’s New Multiple Range Test (Steel and Torrie, 1980) to compare treatment means. Unless otherwise noted, high significance was declared at $P<0.01$, significance was declared at $P \leq 0.05$, and non-significance was declared at $P>0.05$.

**RESULTS AND DISCUSSION**

Daily DM intake for calves fed different CP diets did not significantly (Basra *et al.*, 2003a). In contrast average daily CP intake in calves increased with increasing CP level in the diet (Basra *et al.*, 2003a; 2003b). N excretion through urine and N balance increased significantly due to CP diet and N intake (Mehra *et al.*, 2006). However, fecal N was not significant (Table 1). Therefore, the nitrogen requirement for maintenance for growing swamp buffalo calves is $0.75 \text{ g N/kg BW}^{0.75}$ (Figure 1). Basra *et al.* (2003b) found that protein requirements of male *Nili-Ravi* buffalo calves were 20% lower than dairy cattle calves whereas the protein requirement for growth of *Nili-Ravi* buffalo calves was the same as cattle (Basra *et al.*, 2003a). This study concluded that protein requirements for maintenance for growing swamp buffalo calves is $4.69 \text{ g CP/kg BW}^{0.75}$/d.

Protein requirements can be determined through nitrogen balance studies. In these studies, healthy adult animals should be fed an adequate amount of energy and other nutrients in diets that contain different levels of protein or nitrogen. The minimum protein intakes that will support nitrogen equilibrium are the maintenance requirement. Protein requirements can be determined through the regression equation, the relationship between nitrogen intake and average daily gain. Therefore, the maintenance requirement increases with body size. However, it is difficult to determine the precise protein requirements because protein can be used as a source of energy whenever an animal experiences an energy shortage.

For maintenance nitrogen balance in buffaloes, protein must be provided in a sufficient amount to allow for metabolic fecal losses and provide for growth, production and (or) reproduction (Kearl, 1982). NRC (1996; 2001) reported that metabolic fecal, urinary, and scurf losses represent the requirement needed for maintenance. The maintenance requirement increases with body size and decreases as the animal approaches maturity due to the decreasing protein content in the body tissue. Every animal, regardless of the diet or the physiological function being performed, will have urinary nitrogen losses. This loss is reasonability constant per unit of body size. Fecal losses normally will vary with the composition of the maintenance diet and the metabolic fecal nitrogen. The metabolic fecal portion in the feces contains substances that indicate their origin in the animal’s body such as bacterial residues, cells from the walls of the gastro-intestinal tract and in residues of the digestive juices and other secretions (Kearl, 1982). Although the metabolic fecal nitrogen may be relatively constant in terms of body size and the total fecal nitrogen depends on the digestibility of the dietary protein provided to the animal, it is difficult to measure fecal and urinary losses independently of each other. It is also difficult to separate microbial losses in the feces from true metabolic fecal losses. For most ruminants, metabolizable protein (MP) and crude protein (CP) requirements, using the calculation based on indigestible dry matter intake, are unrealistically high (NRC, 1996). The high requirement can be attributed to the fact that nitrogen is being excreted
Table 1. Effect of dietary protein on nutrients intake, body weight change and N metabolites in swamp buffalo calves.

<table>
<thead>
<tr>
<th>Items</th>
<th>Dietary crude protein levels</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5%</td>
<td>7%</td>
</tr>
<tr>
<td>Body weight change (kg)</td>
<td>0.75a</td>
<td>-0.25a</td>
</tr>
<tr>
<td>Dry matter intake (kg/calf/d)</td>
<td>3.84a</td>
<td>3.888a</td>
</tr>
<tr>
<td>Dry matter intake (g/kg BW(^{0.75}/d))</td>
<td>67.85a</td>
<td>68.34a</td>
</tr>
<tr>
<td>Crude protein intake (g/kg BW(^{0.75}/d))</td>
<td>3.47d</td>
<td>4.85c</td>
</tr>
<tr>
<td>Crude protein intake (g/calf/d)</td>
<td>51.10c</td>
<td>71.01c</td>
</tr>
<tr>
<td>N intake (g/ kg BW(^{0.75}))</td>
<td>0.55d</td>
<td>0.78c</td>
</tr>
<tr>
<td>Urine N (g/d)</td>
<td>6.93b</td>
<td>11.70b</td>
</tr>
<tr>
<td>Fecal N (g/d)</td>
<td>22.71a</td>
<td>23.58a</td>
</tr>
<tr>
<td>N balance (g/ kg BW(^{0.75}))</td>
<td>0.03c</td>
<td>0.16b</td>
</tr>
</tbody>
</table>

Figure 1. Relationship between N balance (g/ kg BW\(^{0.75}\)) and N intake (g/ kg BW\(^{0.75}\)) in buffaloes.
in the feces as microbial protein rather than as urea in the urine as a result of the microbial growth in the postruminal digestive tract. NRC requirements (1996) based on MP requirement state that the CP intake needed can be estimated by dividing the total MP requirement by 0.67, which is based on 80 percent of the MP from microbial protein (MCP) and 20 percent from undegradable intake protein (UIP). The CP required is determined as MP/0.67.

The digestible protein (DP) requirement for maintenance of buffaloes recommended by Kearl (1982) is DP = 2.54 g/kg BW0.75/d.

The metabolizable protein (MP) for maintenance of beef cattle recommended by NRC (1996) is MP = 3.8 g/kg BW0.75/d.

The values of N balance regressed linearly for the determination of dietary N requirement for maintenance (Figure 1). The regression equation between N balance and N intake of buffalo was 0.75 g N/kg BW0.75. These findings are in agreement with previous reports in yearling Thai swamp buffaloes (Tatsapong et al., 2010), in Thai-indigenous heifers (176 g CP/d or 4.5 g CP/kg BW0.75/d) (Chantiratikul et al., 2009), in male Thai native cattle (4.28 g CP/kg BW0.75) (Paengkoum, 2010). The current results for the protein requirement for maintenance are approximately 12% and 14% lower than Kearl (1982) recommendation for growing domestic buffaloes (5.24 g CP/kg 4.28 g CP/kg BW0.75) and growing crossbred cattle (5.36 gCP/kg BW0.75). However, the current results are in an agreement with Basra et al. (2003a) and Tauqir et al. (2009a) who found that the protein requirements of Nili-Ravi buffalo calves were lower than the dairy calf recommendation by the NRC (2001). In contrast, Tauqir et al. (2009b) suggested that the CP requirements of Nili-Ravi buffalo calves were higher than those recommended by the NRC (2001) for dairy cattle. Buffaloes seem to have a lower requirement for protein than cattle; the reason for this may be that buffaloes use a greater proportion of the OM for biomass production at the expense of VFAs compared with cows (Calabro et al., 2008). Protein requirement for maintenance of animals appears to depend on climatic conditions (Marai and Haeeb, 2009), breed, mature body size, composition of body tissue, feed quality and growth rate (Kearl, 1982; NRC, 1996).

**CONCLUSION**

From this study it can be concluded that increasing dietary nitrogen in buffalo diets significantly increased (P<0.05) N intake and N retention or N balance. The present findings suggest that the protein requirements for maintenance of growing Thai swamp buffalo were 4.68 g CP/kg BW0.75/d or 0.75 g N/kg0.75/d. However, further studies should be conducted for the validation of nutrients requirement (especially protein and energy) for different physiological stages of buffaloes. To optimize energy and protein efficiency and to reduce nitrogen wastage, diets need to be formulated to provide optimum fermentable energy sources and nitrogen concentration for maximum rumen microbial yield and growth.

**ACKNOWLEDGMENTS**

The authors acknowledge the Suranaree University of Technology (SUT) and the National Research Council of Thailand (NRCT) for financial and facility support of this research.
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*Continued on page 52
SUPPRESSION OF THE CELLULAR RESPONSE IN BUFFALO CALF SKIN BY CYPROHEPTADINE DRUG

Neelu Gupta¹, A.K. Katiyar² and Madhu Swamy³

ABSTRACT

This study was conducted on 3-6 month old 12 buffalo calves. The buffalo calves were randomly divided into two groups- a control group (1) and an experimental group (2) Each group were divided into subgroups i.e. 1A,1B and 2A, 2B respectively. Experimental calves were pretreated with cyproheptadine intramuscularly 30 minutes prior to intradermal injection of Staph. Epidermis (Group 2A) and turpentine (Group 2B). Lesions of different time intervals were obtained for the sequential study of cellular responses. Maximal suppression of leukocytes was observed at 3 h in both types of inflammation. In both subgroups neutrophils were markedly suppressed at 3 h as compared to others cells.

Keywords: buffalo calf, Bubalus bubalis, cellular response, Staphylococcus epidermidis, turpentine

INTRODUCTION

In the buffalo, few reports are available on the bacteriological, epidemiological and cellular responses in clinical and subclinical spontaneous cases of mastitis (Chaudhry et al., 1982; Muhammad et al., 1996). However, the information on the cellular responses occurring in experimentally induced inflammation in the buffalo seems to be lacking in the literature. Further, the mediation of the inflammatory response has been studied by number of workers using the anti-inflammatory drugs in experimental animals and birds. However, this aspect in the buffalo has also remained unexplored. Thus, this work was done on buffalo calves to study the suppression of cellular response in buffalo calf skin by cyproheptadine in response of the chemical (turpentine) and bacterial injury (Staphylococcus epidermidis).

MATERIALS AND METHODS

Healthy male buffalo calves (12), 3-6 month-old were divided into two groups: a control and an experimental group, for the study of the cellular response in the buffalo calf skin. Each group comprised six calves. All calves were maintained under standard hygienic conditions. The site of the cutaneous reaction was prepared according Zarrilli and Calhoun (1970). Control Group 1 - The six calves of the control group were again equally divided into two subgroups, i.e., Subgroup 1A and Subgroup 1B. Each subgroup had three calves.

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Each calf of Subgroup 1A received two intradermal injections of *Staphylococcus epidermidis* (0.1 ml) suspension in normal saline and each calf of subgroup 1B received two intradermal injections of turpentine for each time interval of 0-2 minutes, 30 minutes, 1 h, 3 h, 6 h, 12 h, 24 h and 48 h on both side (left and right) of thoraco-abdominal region. Each calf received in all sixteen injections, two for each time interval and making six lesions per time interval. The calves were euthanized by saturated solution of magnesium sulphate given intravenously.

**Experimental Group 2** - Six calves of experimental group were again equally divided into two subgroups, i.e., Subgroup 2A and Subgroup 2B. Each calf of Subgroup 2A was pretreated with cyproheptadine i/m at the dose rate of 1 mg/kg body weight 30 minutes prior to i/d *Staph epidermidis* (0.1 ml) and this was repeated every 12 h. The rest of the procedure was same as in control group. The skin specimen were collected and fixed in Cornoy’s fluid for histopathological studies as described for chicken by Shrivastava *et al.* (1997). Sections were cut at 4-5 μm thickness. Skin sections were stained with haematoxylin and eosin, and with 0.05 percent solution of toluidine blue in acetate buffer (pH 3-8) for basophils as described for chickens by Dhodapkar *et al.* (1984).

## RESULTS AND DISCUSSION

**Control Group 1A** - Vesicular changes were noticed at initial stage (0-2 minutes, and 30 minutes) Hyperaemia of blood vessels and oedema of the dermis were marked at 1 h and gradually increased. More marked hyperaemia and extensive oedema were noticed at 6 h. Necrosis of dermis was noticed at 24 h. Leukocyte emigration was also noticed at the 30 mininterval and gradually significantly increased up to 12 h and then the number of cells decreased considerably and no cells were noticeable at 48 h in the buffalo skin. The maximal infiltration of neutrophils was at 6 h, mononuclear cells at 12 h, lymphocytes at 24 h basophils at 1 h and total leukocytes were observed at 12 h. Infiltration of eosinophils was not noticed in any one stage (Table 1).

**Subgroup 1B**-Vascular changes of blood vessels and oedema of dermis were not evident at the 0-2 minutes time interval. These changes were observed from 30 minutes onward. More marked hyperemia and oedema were observed at 6 h. Leukocyte migration was noticed at 0-2 minutes along with few neutrophils in small blood vessels. The significantly highest number of neutrophils, monocytoids, lymphocytes and basophils were observed at the 6, 12, 48 and 3 h time intervals, respectively. Infiltration of eosinophils was not noticed at any time (Table 2)

**Experimental Group 2- Subgroup 2A**- Thirty minutes before injection of the *Staph epidermidis* suspension in the skin, the calves were pretreated with cyproheptadine intramuscularly and the lesion as per non-pretreated group were obtained and processed for histopatholgical examination.

Cellular changes were not noticed at the initial stage (0-2 minutes, and 30 minutes). The hyperaemia of the blood vessels and oedema of the dermis were marked at 1 h and gradually increased. More marked hyperaemia and extensive oedema were noticed at 6 h but less marked as compared to Subgroup 1A. The maximal suppression of leukocytes was observed at 3 h. A few neutrophils were seen intravascularly at 0-2 minutes; the number of neutrophils increased significantly gradually up to 6 h and revealed maximal rate of suppression being 29.39 percent at 3 h. Monocytes,
Table 1. Tissue leukocytosis in response to *Staphylococcus epidermidis* in control and cyproheptadine pretreated buffalo calves.

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Neutrophils</th>
<th>Monocytoids</th>
<th>Lymphocytes</th>
<th>Basophils*</th>
<th>Total leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 min</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>30 min</td>
<td>0.433 ±0.221</td>
<td>0.400 ±0.105</td>
<td>7.621</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>1 h</td>
<td>10.600 ±0.992</td>
<td>8.366 ±0.991</td>
<td>21.075</td>
<td>1.533 ±0.564</td>
<td>1.400 ±0.149</td>
</tr>
<tr>
<td>3 h</td>
<td>30.166 ±1.996</td>
<td>21.300 ±1.870</td>
<td>29.390</td>
<td>3.033 ±0.784</td>
<td>2.600 ±0.623</td>
</tr>
<tr>
<td>6 h</td>
<td>59.466 ±4.676</td>
<td>47.600 ±3.914</td>
<td>19.995</td>
<td>15.766 ±1.579</td>
<td>12.133 ±0.735</td>
</tr>
<tr>
<td>12 h</td>
<td>50.266 ±1.206</td>
<td>42.133 ±2.529</td>
<td>16.179</td>
<td>27.366 ±1.883</td>
<td>21.160 ±1.257</td>
</tr>
<tr>
<td>24 h</td>
<td>10.066 ±3.023</td>
<td>9.5 ±1.143</td>
<td>5.622</td>
<td>5.066 ±1.530</td>
<td>4.566 ±0.571</td>
</tr>
<tr>
<td>48 h</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
</tr>
</tbody>
</table>

*Toluidine blue sections*
Table 2. Tissue leukocytosis in response to turpentine in control and cyproheptadine pretreated buffalo calves.

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
<th>Basophils*</th>
<th>Total Leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent Suppression %</td>
<td>Number of Leukocytes (Mean ± S.E.) / high power(x400) microscopic field</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Turpentine</td>
<td>Cyproheptadine</td>
<td>Turpentine</td>
<td>Cyproheptadine</td>
<td>Turpentine</td>
</tr>
<tr>
<td>0.2 min</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>30 min</td>
<td>10.200 ±1.206</td>
<td>7.633 ±0.705</td>
<td>15.181</td>
<td>0.433 ±0.194</td>
<td>0.400 ±0.210</td>
</tr>
<tr>
<td>1 h</td>
<td>24.500 ±1.336</td>
<td>17.066 ±3.325</td>
<td>30.34</td>
<td>2.300 ±0.699</td>
<td>2.060 ±0.434</td>
</tr>
<tr>
<td>3 h</td>
<td>137.996 ±6.762</td>
<td>78.566 ±1.934</td>
<td>43.054</td>
<td>18.300 ±2.267</td>
<td>15.933 ±0.880</td>
</tr>
<tr>
<td>6 h</td>
<td>244.833 ±5.002</td>
<td>148.133 ±3.522</td>
<td>39.496</td>
<td>71.530 ±2.837</td>
<td>50.900 ±2.561</td>
</tr>
<tr>
<td>12 h</td>
<td>196.866 ±3.32</td>
<td>141.733 ±0.730</td>
<td>28.005</td>
<td>95.833 ±4.980</td>
<td>75.100 ±1.642</td>
</tr>
<tr>
<td>24 h</td>
<td>134.600 ±2.995</td>
<td>111.266 ±3.707</td>
<td>17.335</td>
<td>72.566 ±3.089</td>
<td>67.566 ±1.240</td>
</tr>
<tr>
<td>48 h</td>
<td>94.933 ±4.086</td>
<td>88.433 ±1.710</td>
<td>6.846</td>
<td>48.633 ±2.466</td>
<td>46.766 ±1.627</td>
</tr>
</tbody>
</table>

*Toluidine blue sections
lymphocytes and basophils cells showed maximum suppression at 6 h, 3 h and 1 h respectively (Table 1).

**Subgroup 2B-** Thirty minutes before injection of turpentine in the skin, the calves were pretreated with cyproheptadine intramuscularly, and the lesion as per non-pretreated group were obtained and processed for histopathological examination.

Vascular changes of the blood vessels and oedema of dermis were not evident at 0-2 minutes time interval. These changes were observed from 30 minutes onward. More marked hyperemia and oedema were observed at 6 h but less than Subgroup 1B. The significantly maximal suppression of leukocytes, neutrophils, lymphocytes and basophils was recorded at 3 h while maximal suppression of monocytes was noticed at 6 h (Table 2).

The emigration of the leukocytes was suppressed in cyproheptadine pretreated calves in *Staphylococcus epidermidis* and turpentine-induced inflammations. Maximal suppression of the leukocyte infiltration was recorded at 3 h in both stimuli. In bacterial inflammation cyproheptadine caused maximal suppression of the neutrophils at 3 h, monocytes cells at 6 h, and lymphocytes and basophiles at 1 h. Whereas, in turpentine injury, maximal suppression of the neutrophils, lymphocytes and basophiles at 3 h, and of monocytes at 6 h interval. In both stimuli neutrophils were more extensively suppressed as compared to mononuclear cells. The effect of non-steroidal anti-inflammatory drugs on cell migration has not been extensively investigated. Furthermore, to our knowledge, the selected inhibition of 5-HT by use of its antagonist and the resultant effect on cellular response remains totally unexplored in mammals. However, Khare (2000) studied the suppression of cellular response in birds pretreated with reserpine, a known 5-HT antagonist. The worker reported a significant suppression of leukocyte infiltration at site of punch wounding. However, in birds, monocytooids cells were reduced in greater number as compared to heterophils. The findings differed from our observations in buffalo, where the infiltration of neutrophils was suppressed more than that of monocytes cells. Gupta *et al.* (2007, 2008) reported in the buffalo, antihistamine also suppressed neutrophil infiltration more significantly than monocytes and leukocytes taken together. It can be suggested that antagonists of vasoactive amine have more suppressive effect on neutrophil infiltration in buffaloes. However, further investigation needs to be in conducted.

**REFERENCES**


ABSTRACT

The aim of this study was to evaluate the effects of the addition of different levels of antioxidant (taurine) on buffalo spermatozoa cryopreserved in an egg yolk based extender. Ejaculates were collected from five mature buffalo bulls (four ejaculates per buffalo) and were evaluated and diluted at 37°C in one of the following experiments: tris-egg yolk extender (control), or the same extender supplemented with either 10, 25, 50, 75 or 100 mM taurine. The semen was loaded into 0.5 ml straws, cooled and frozen in a programmable freezer and subsequently stored in liquid nitrogen. Prior to evaluation, frozen straws were thawed in a water bath (37°C for 20 s). Freezing extenders supplemented with 25 and 10,100 mM taurine led to higher and lower sperm motility values (respectively), compared to the control (P<0.05) and following the freeze-thawing process. The addition of antioxidants did not affect acrosomal integrity compared to the control.

Keywords: buffaloes, Bubalus bubalis, semen, extender, taurine, microscopic parameters

INTRODUCTION

The buffalo is second-ranked milk producer in world and produces more than a third of Asian milk (Bandyopadhyay et al., 2003). About 23% of Iran’s buffalo are in Azerbaijan Province. Buffalo nurturing is difficult because of the innate susceptibility of this animal to environment tensions that cause mild heat and quiescent heat and increase days open and cause heavy losses to the buffalo husbandry industry (Ingawale et al., 2004). The increasing importance of buffaloes has led to the introduction of artificial insemination to increase the breeding efficiency of this species. One of the important characteristics of mammalian sperm is its high density of unsaturated fatty acids on phospholipids structures that are distributed asymmetrically throughout the lipid layers of the plasma membrane (Aitken et al., 1993; Aitken, 1995 and Gadella et al., 1999). Buffalo sperm is more susceptible to cold stress than that of other species such as bull, rabbit and human (Fise et al., 1989 and Watson., 1981). Of course, different reactions to temperature tensions results from differences in membrane lipid composition as buffalo sperm has high levels of saturated and unsaturated fatty acids and has lowest ratio of clostrol/phospholipid compared with other species (Evans, 1988). The low fertility of frozen semen compared with fresh
semen and natural mating is because of injuries due to freezing and thawing, and in a comparison of frozen semen with fresh semen of buffalo, about 8 times more frozen semen was required to achieve the best fertility (Shannon et al., 1995). The motility of frozen-thawed semen is lower in the buffalo than in the bull: in the buffalo, it is about 10-20 percent whereas in the bull, it is 30-35 percent (Nandi et al., 2006 and Totey et al., 1992). Sperm cells are very susceptible to peroxidation of membrane lipids after thawing, because they have high amounts of unsaturated fatty acids in their structures which cause metabolic alterations and loss of special sperm functions such as motility, solidarity of sperm membrane, and fertility (Alvarez et al., 2005; Cassani et al., 2005; Lenzi et al., 2002; Storey, 1997 and Wishart, 1984). During the freezing through of the membrane, unsaturated fatty acids peroxidation produces reactive oxygen (Alvarez et al., 2005 and Jones et al., 1997). Addition of amino acids to the extender has positive effects on motility after thawing of sperm and protection of sperm membrane integrity in different species (Chen et al., 1993; Pena et al., 1998 and Sanchez et al., 1997). Taurine and hypotaurine are required for capacitation and fertility of sperm (Guerin et al., 1995). Positive effects of taurine on protection of membrane function and structural integrity of the acrosome membrane has been established (Aruoma et al., 1998; Fellman et al., 1985 and Pasantes et al., 1989).

MATERIALS AND METHODS

Materials

All materials used in this study such as taurine were supplied from the Sigma Company.

Animals and semen collection:

Five mature buffalo bulls (3-4 years old) were selected in the Northwest Buffalo Research and Nurturing Center. Collection of semen samples was done in the spring by artificial vagina and after teasing of selected buffalo bulls via a constricted female buffalo. The 20 ejaculations of the five native buffalo bulls were collected (four replications for each animal and the volume of each ejaculation was 2 ml) for twice in one week.

Fresh and post-thawing semen samples evaluation:

Rapidly after collection of the samples, the semen was transported to the lab and primary evaluation was done of concentration and motility aspects. One semen droplet was placed on a slide and sperm motility percentage was assayed by hot plate microscope (200×).

Also sperm concentration of each ejaculation was designated via spectrophotometer. Those ejaculations were selected which had 1-2 ml volumes, sperm motility more than 70% and concentration more than 2.5x10⁹ sperm per ml. In this study, we used tris as basic extender. Collected semen samples were divided into 11 identical portions, and at 37°C were mixed with basic extenders concommitant with five levels of taurine (10, 25, 50, 75 and 100 mM) and basic extender without antioxidant (final sperm concentration was 5x10⁶ sperms/ml). Diluted semen samples were transferred to 0.5 ml straws and were sealed with polyvilin alcohol powder and then frozen. After one month, from each group, five straws were selected randomly and thawed for 20 seconds at 37°C in a water bath. Percentage motility was assessed using a phase-contrast microscope (x40). After supravital staining, viability was evaluated by microscope with 400x magnification as a second index of
sperm quality. For assessment of acrosome health and natural head structure, 500 μl of thawed semen samples was mixed with 50 μl citrate formaldehyde 1% and citrate tri sodium dehydrate 2.9% to fix sperms. After fixing, prepared slides were assessed through a phase-contrast microscope.

Statistical analysis:
The study was replicated three times. Results are expressed as the mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used to assess differences among treatments on motion characteristics, sperm viability and normal acrosome morphology. When the F ratio was significant ($P<.05$), Tukey’s post hoc test was used to compare treatment means (Version 12.0; SPSS, Chicago, IL).

RESULTS

Sperm parameters
sperm motility: As shown in Table 1, among treatments, maximum motility were achieved in 25 mmol of taurine and minimum were achieved in 10 and 100 mmol of taurine.
sperm acrosomal integrity: Among treatments, best acrosomal condition was seen in control group but, differences between groups was not significant.
sperm viability: According to Table 1, about antioxidant treatments, treatment of 75 mmol of taurine had maximum increasingly effect on viability percentage of sperm after thawing and increasing of antioxidant amounts for more than 50 mmol has negative effects on sperm viability.

DISCUSSION

Improvement of semen freezing methods requires knowledge about biochemical and physiological processes that occur during the freezing, thawing and extender characteristics (Holt., 1997; King et al., 2004 and Sariozkan et al., 2009). Antioxidant capability in sperm cells is limited because of a deficiency cytoplasmic components having antioxidant effects to expunge

Table 1. Effect of different concentrations of Taurine on buffalo-bull semen characteristics after freezing-thawing.

<table>
<thead>
<tr>
<th>Live sperm (%)</th>
<th>Acrosomal integrity (%)</th>
<th>Motility (%)</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>52.77 ± 0.77de</td>
<td>68.33 ± 0.23</td>
<td>46.72 ± 0.38c</td>
<td>Taurine 10 mM</td>
</tr>
<tr>
<td>65.22 ± 0.75b</td>
<td>68.52 ± 0.29</td>
<td>58.13 ± 0.17a</td>
<td>Taurine 25 mM</td>
</tr>
<tr>
<td>73.98 ± 0.62a</td>
<td>67.57 ± 0.33</td>
<td>56.90 ± 0.19a</td>
<td>Taurine 50 mM</td>
</tr>
<tr>
<td>59.48 ± 0.98c</td>
<td>68.50 ± 0.23</td>
<td>53.41 ± 0.22b</td>
<td>Taurine 75 mM</td>
</tr>
<tr>
<td>53.77 ± 0.49d</td>
<td>67.07 ± 0.27</td>
<td>46.80 ± 0.42c</td>
<td>Taurine 100 mM</td>
</tr>
<tr>
<td>52.13 ± 0.40e</td>
<td>68.63 ± 0.09</td>
<td>45.08 ± 0.11d</td>
<td>Control</td>
</tr>
</tbody>
</table>

Values are (mean ± standard error of mean). Different letters within a column indicates significant differences ($P<0.05$).
reactive oxygen. Thus, mammalian sperm does not have enough ability to counter peroxidation during the freezing and thawing processes (Alvarez et al., 2005; Bilodeau et al., 2000 and Lapointe et al., 2003). Reactive oxygen may be responsible for the loss of motility, acrosomal membrane integrity, and fertility and for sperm metabolic alterations. In recent years, adding antioxidants to semen extenders for improvement of sperm quality have been studied. Addition of antioxidants with maintenance of acrosome and mitochondrial integrity against cold increases post-thawing sperm motility. Viability and acrosomal membrane continuity are important in evaluation of semen quality, because the sperm motility test is not sufficient for evaluation of viability after freezing and thawing. Improvement of semen parameters after adding antioxidants (such as taurine and trehalose) to extenders in bull, pig, sheep, goat and dog has been reported. Taurine acts as non enzymatic antioxidant which have important role in protecting sperms against reactive oxygen species. Eleshestavi et al., 2008 were reported that adding special amino acids (glutamine, glycine, alanine and systein) to extenders (tris, citrate, fructose and glycerol) before freezing, causes improvement of semen quality after thawing. In the current study, addition of different levels of the antioxidant to the extender before freezing caused significant improvement in sperm quality such as forward motility and viability of sperms compared to the control group (P<0.05) but no significant differences in acrosomal integrity between treatment and control groups were seen. Only in the t 100 mmol taurine treatment was a significant difference in acrosomal health seen: 100 mmol of taurine caused significant decreases in acrosomal integrity and finally decreasing fertility. The results achieved in this study in improvement of sperm motility after thawing were similar with results achieved in sheep, rabbit and pig sperms (Atessahin et al., 2008; Bucak et al., 2007 and Molinia et al., 1994). On the other hand, adding taurine to extenders has led to no improvement in motility in bull and goat sperm after thawing, probably because of species diversity, extender composition and antioxidant density. Taurine has a protective effect against lipid peroxidation and losing motility in ram sperm and also has positive effects on viability and membrane continuity of ram sperm after thawing. Chen et al., 1993, Pena et al., 1998 and Sanchez et al., 1997 reported that sperm motility significantly increased with the addition of antioxidants to extenders after thawing; this is compatible with the current research results but is inconsistent with our research results with regard to acrosomal condition. Some studies revealed that adding taurine and cysteine to extenders before freezing causes improvement in forward motility of sperm after thawing in pig, bull, human, sheep and goat that is compatible with our research results (Atessahin et al., 2008; Bucak et al., 2008; Funahashi et al., 2005; Uysal et al., 2007).

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ABSTRACT

A technique for isolation of leucocytes (neutrophils, macrophages and lymphocytes) and epithelial cells from buffalo milk was successfully carried out. Higher fat and total solids in buffalo interfere with the isolation of milk cells, but an increase in the duration and speed of centrifugation helps in the successful isolation of these cells. Isolation of epithelial and milk leucocytes from milk is a non-invasive, easily repeatable technique which allows frequent sampling and can be used to study the immune and synthetic activity of exfoliated milk cells in vitro.

Keywords: buffalo, milk, neutrophils, macrophages, lymphocytes, epithelial cells

INTRODUCTION

Milk somatic cells are simply animal body cells present at low levels in normal milk. These cells include both the leukocytes and the epithelial cells which are sloughed off during the normal process of milking throughout the lactation cycle (Harmon, 1994). The epithelial cells are capable of synthesizing milk, whereas, the white blood cells serve as a defense mechanism to fight disease (infection), and assist in repairing damaged tissue. Various leucocytes and epithelial cells can be isolated and studied for their activity from the milk cell pellet after centrifugation at low speed (Boutinaud and Jammes, 2002). After isolation, milk somatic cells can also be used for dynamic studies of gene expression in the mammary gland. Isolation, culture and determination of activity of each milk leucocyte can also help in assessing mammary gland immunity and may be used as a tool for genetic selection of high-producing animals. Successful isolation and culture of neutrophils from colostrum and milk from buffaloes has been developed (Dang et al., 2010). But there is no report on the isolation of milk lymphocytes, macrophages and epithelial cells from milk of buffaloes, which are the major contributors of milk production in South Asia. Also, buffalo milk is different from that of cows as it has more fat and total solids, which prevent proper isolation and culture of milk cells. Therefore, the main objective of the present study was to isolate and culture exfoliated cells from the milk of buffaloes.

MATERIALS AND METHODS

Milk samples were collected from healthy Murrah buffaloes having normal somatic cell counts (Dang et al., 2010). Udder and teats of selected buffaloes were washed and cleaned with 70% absolute alcohol. Milk was collected in...
sterilized containers and processed within 2 h of milk sampling.

**Isolation of polymorphonuclear neutrophils from milk**

For isolation of polymorphonuclear neutrophils, fresh milk was filtered through a nylon filter (40 μm pore size) and diluted to 60% with cold Dulbecco’s PBS (volume/volume). Isolation of PMN was performed using four centrifugation steps as buffalo milk is higher in milk fat. Ten milliliters of milk was poured into a centrifuge tube and centrifuged (800X g, 20 minutes, 4°C). The fat was removed with supernatant. The remaining cell pellet was washed thrice in cold Dulbecco’s PBS (500X g, 10 minutes, 4°C, 300X g, 20 minutes, 4°C and finally at 300X g, 20 minutes, 4°C). The final pellet was resuspended in Dulbecco’s PBS containing 0.5 mg/ml gelatin. Addition of gelatin maintains the integrity of the neutrophils.

**Isolation of lymphocytes from milk**

Isolation of lymphocytes from milk was done by density gradient centrifugations. Milk was centrifuged (600X g, 20 minutes, 4°C) and the fat layer accumulated on top was removed along with the skim milk. The cell pellet obtained at the bottom was dissolved in PBS. This was layered over the Histopaque -1077 and was centrifuged at 600X g for 20 minutes at 4°C. The fat layer on top was removed and the whole white layer of lymphocytes above the Histopaque was collected in which lymphocytes comprised the major portion of DLC (69-71%), whereas, monocytes were found to range between 1-3%. It was washed once with PBS and finally once with media.

**Isolation of macrophages from milk**

For isolating macrophages, milk was placed into sterile 50-ml siliconized tubes and diluted 1:4 with PBS and then centrifuged at 4°C and 500X g for 15 minutes. The supernatant milk was recentrifuged to ensure maximum yield of cells per sample, and the pellets were combined. Cells were layered onto Ficoll-Hypaque and centrifuged at 500 X g for 30 minutes. The mononuclear cell layer was washed twice with PBS and suspended in Dulbecco’s modified Eagle’s medium containing penicillin and streptomycin. Cells were allowed to adhere to tissue culture dishes for 1 h at 37°C. Adherent cells were washed vigorously twice with HBSS. After the adherence, cells were assayed immediately or incubated overnight in medium. Adherent cells were found to be 90 to 95% macrophages by May-Grünwald stain.

**Isolation and culture of epithelial cells from milk**

Briefly, milk samples were collected from buffaloes and centrifuged at 3500 rpm, 20°C for 15 minutes. The supernatant was discarded and the remaining milk cell pellet was resuspended in buffer. The milk was again centrifuged for 5 minutes at 3000 rpm. The supernatant was discarded and the remaining milk cell pellet was again resuspended in buffer. The cell pellet was resuspended in Dulbecco’s Modified Eagle’s Medium and filtered through a nylon cell strainer. After another centrifugation at 3000 rpm, the remaining cell pellet was resuspended in 10 ml pre-heated medium (37°C). The medium with the cells were kept in sterile tissue culture bottles. The medium was changed twice per week. The cells appear to be inert for the first two days of culture, after which both small and large cells adhered to plastic dishes, making it possible to distinguish them from lymphocytes and granulocytes, which remained in suspension.
RESULTS AND DISCUSSION

The present study was conducted for the first time to isolate milk macrophages, neutrophils, lymphocytes and epithelial cells from milk of buffaloes. The results of the smears of milk neutrophils, lymphocytes and macrophages have been presented in Figures 1, 2 and 3, respectively. Whereas, the results obtained after culturing of milk neutrophils, lymphocytes, macrophages and epithelial cells have been presented in Figures 4, 5, 6 and 7, respectively. All the culture photographs were taken at 1000 X magnification under oil immersion. The viability of all milk leukocytes was determined using Trypan Blue, after counting the cells using a hemocytometer. The isolation procedure of PMN from milk yielded >80% of granulocytes (PMN + eosinophils) with predominantly PMN (>80%) as determined by counting the cells in smears stained with Leishman’s stain. The viability of milk lymphocytes in different experiments was found to range between 95-98% within 6 h of processing and declined gradually afterwards. The viability of milk macrophages was found to be around 91-93% after washing and declined afterwards. Very few live epithelial cells were present in buffalo milk, but all these cells were able to proliferate and terminally differentiate into small colonies. The presence of mammary epithelial cells in the culture was confirmed further by immunostaining. On day 30, three different cell colonies of epithelial cells were seen; tightly joined elongated cells, tightly joined cuboidal cell colonies and contiguous cells were observed.

Our results indicate that as in cows (Mukherjee and Dang, 2011) various cells can also be isolated from buffalo milk after increasing the duration and number of centrifugation steps. Further, by isolating milk phagocytes, i.e. both macrophages and neutrophils, and challenging them with bacteria, their phagocytic activity can be estimated as is done for blood neutrophils (Dang et al., 2007). Lymphocyte proliferation assay can be studied from the isolated milk lymphocytes by stimulating them with various mitogens as done for blood lymphocytes. Also live epithelial cells could also be harvested from buffalo milk as reported in cow’s milk (Buehring, 1990) and used for determining their synthetic and immune activity.

Of all the leucocytes, neutrophils are the first ones to respond (Jain, 1986) and migrate from blood circulation to an inflamed area where they phagocytose and kill bacteria. As neutrophils travel from the blood to the mammary gland their activity is reduced which increases the chances of intra mammary infections (Paape et al., 2002). This is because milk neutrophils have reduced abilities to produce reactive oxygen species, when compared to blood (Goldbery et al., 1995; Dosogne et al., 2001). Also diapedesis of blood PMN through the blood-milk barrier causes a reduction of the phagocytic and oxidative burst activity was also demonstrated using an in vitro cell culture model (Smits et al., 1999). Therefore, for selection of high-producing buffaloes we can isolate individual neutrophils, macrophages and lymphocytes and estimate their immune activity. Only buffaloes having higher production potential along with improved immune function should be selected. Selection done on this basis may reduce the incidence of subclinical or clinical mastitis in high-producing buffaloes.

Above all, the above technique of isolation of milk somatic cells is a non-invasive technique. In the future, this technique not only will help us in the better understanding of the immune regulation in the mammary gland but may also lead to improved treatments and vaccine development for our high-producing buffaloes.
Figure 1. Milk neutrophils (40X).

Figure 2. Milk lymphocyte (40X).

Figure 3. Milk macrophage (100X).
Figure 4. Culture of milk neutrophils (6 h) (1000X).

Figure 5. Culture of milk lymphocytes (12 h) (1000X).

Figure 6. Culture of milk macrophages (24 h) (1000X).
REFERENCES


Figure 7. Culture of milk epithelial cells (45 days) (1000X).
ABSTRACT

Eighteen buffaloes selected from a private dairy farm of the Ludhiana region were divided into three groups of six animals each. The first group served as healthy control, the second and third as treatment groups. Treatment was given on the $7^{th}$ day of observation with injRedoxin 20 ml I/M s.i.d for 5 days in Group 2 and with inj Vetade 10 ml/500 kg b.wt. s.i.d. for 4 days in Group 3. Vitamin C and vitamin E levels were assayed on the $0^{th}$, $7^{th}$, $14^{th}$ and $21^{st}$ days of observation. In Group 1, the levels of vitamin C, vitamin E and selenium were $1.0795 \pm 0.019$ to $1.088 \pm 0.025$ mg/dl, $3.983 \pm 0.064$ to $3.946 \pm 0.133 \mu$mol/l and $0.611 \pm 0.059$ to $0.62 \pm 0.072 \mu$g/ml, respectively. Pre-treatment and post-treatment value values of vitamin C were $0.765 \pm 0.128$ to $1.0795 \pm 0.019$ mg/dl, respectively. In Group 2, showing a significant difference, i.e. a 28% increase in the level after treatment. Pre-treatment and post-treatment values of vitamin E ranged from $2.58 \pm 0.24$ to $3.006 \pm 0.434 \mu$mol/l and $3.983 \pm 0.064$ to $3.946 \pm 0.133 \mu$mol/l, respectively, in Group 3, showing a significant difference, i.e. a 41% increase in the level. Pre-treatment and post-treatment values of selenium ranged from $0.19 \pm 0.062$ to $0.245 \pm 0.068 \mu$g/ml and $0.690 \pm 0.025$ to $0.695 \pm 0.031 \mu$g/ml, respectively, showing a 2.18 % increase in the level. Eight animals out of the 12 were cured suggesting that antioxidant containing drugs can act as an adjunct therapy in the treatment of mastitis.

Keywords: mastitis, redoxin, vetade, vitamin C, vitamin E, selenium

INTRODUCTION

Buffaloes are the preferred dairy animal in rural India, contributing over 50% of the total milk production in the country. Unhygienic and unscientific milking practices and hot-humid environmental conditions predispose them to mastitis. The loss of US$ 8.80 per buffalo per lactation due to mastitis speaks of its tremendous economic losses due to reduced milk production without considering the cost of disease management (Thirunavukarsu and Prabaharan, 2000). In India, total annual losses due to mastitis (clinical and subclinical) in buffaloes have been estimated at US$526million (Dua, 2001). Antibiotic therapy is not only costly, but also poses residue problems in milk adversely affecting human health (Hoeben

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et al., 1997). The cure rate with antibiotics is approximately 60% under field conditions. When β-lactamase producing organisms are implicated, the cure rate becomes poorer still (Radostits et al., 2000). Treatment failure with available allopathic drugs in fibroed quarters also poses challenges to veterinarians. Therefore, the search for cost effective complementary and alternative treatment approaches for management of mastitis is being pursued throughout world.

Deficiency of many vitamins and micronutrients particularly vitamin A, vitamin D, vitamin E, selenium, copper in the diet leads to increased incidence of mastitis with infection of longer duration and more severe clinical signs. For lactating dairy animals, nutrient supplementation for trace minerals and vitamins goes beyond correcting for deficiencies; it is aimed rather at minimizing stress and optimizing production efficiency (McDowell, 2002).

Free radicals can be extremely damaging to biological systems (Padh, 1991). Also, phagocytic granulocytes undergo respiratory bursts to produce oxygen radicals to destroy intracellular pathogens. However, these oxidative products can, in turn, damage healthy cells if they are not eliminated. Antioxidants serve to stabilize these highly reactive free radicals, thereby maintaining the structural and functional integrity of cells (Chew, 1995). Therefore, antioxidants are very important to immune defense and health of animals.

Tissue defense mechanisms against free radical damage generally include vitamin A, vitamin C, vitamin E and some trace minerals like selenium. These are also critical in protecting the internal cellular constituents from oxidative damage. Both in vitro and in vivo studies show that these nutrients generally enhance different aspects of cellular and non-cellular immunity. So, the antioxidant function could at least in part enhance immunity by maintaining the functional and structural integrity of important immune cells. A compromised immune system will result in reduced animal production efficiency through increased susceptibility to diseases, thereby leading to increase animal morbidity and mortality. Antioxidant supplementation could decrease the duration, incidence and severity of clinical mastitis (Erskine et al., 1989; Smith et al., 1984) and was associated in lower prevalence of intra-mammary infections caused by contagious pathogens (Erskine et al., 1987).

Keeping the above facts in view, this study was planned to evaluate the therapeutic efficacy of anti-oxidants like vitamin C, vitamin A, vitamin E, and vitamin D₃ in recurrent cases of mastitis in buffaloes.

**MATERIALS AND METHODS**

Eighteen buffaloes selected from a private dairy farm of the Ludhiana region were divided into three groups of six animals each. Group 1 served as a healthy control, Groups 2 and 3 as treated groups. Treatment was given on the 7th day of observation with inj Redoxin* 20 ml I/M s.i.d for 5 days in Group 2 and with inj Vetade** 10 ml/500 kg b.wt. s.i.d for 4 days in Group 3. Pre-treatment and post-treatment blood sampling was done on the 0th, 7th, 14th and 21st day of observation.

*Inj. Redoxin containing vit. C. Each 5 ml contains 500 mg of ascorbic acid. Ramson remedies, 186, Industrial focal point, Amritsar.A.O. 2, G Floor, Baktawar Malabar Hill, Mumbai-6. Dose - 20 ml i/m s.i.d. for 5 days.

**Vit. ADE₃ injection. Each ml contains vit. A - 250000 IU, Vit.D₃ - 25000 IU and Vit. E -
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100 IU. Marketed by Sarabai Chemicals, Animal Health Ltd. Administration Building, Gorwa Road, Vadodhara-390023, India. Dose - 10 ml/500 kg b.wt. s.i.d. for 4 days.

Collection of whole blood

30 ml of blood was collected in heparinized vials (1:10,000) from each animal suffering from clinical/subclinical mastitis. Twenty milliliters of blood was centrifuged at 3000 rpm for 10 minutes to collect plasma for estimation of vitamin C (estimated within 24 h) and vitamin E (estimated within 48 h) and 10 ml of blood was kept in a deep freeze for estimation of selenium.

Estimation of vitamin C

Vitamin C was estimated by the 2, 4-dinitrophenyl hydrazine (DNPH) method as described by Baker and Frank (1968). This method is based upon principle that coupling of 2, 4-dinitrophenyl hydrazine to the keto groups of carbon 2 and 3 of diketogluconic acid yields an osazone called bis-2, 4-dinitrophenyl hydrazone. In strong acid, this osazone rearranges to a stable reddish brown product, which is measured photometrically. In this method, 2, 6-dichlorophenolindophenol oxidized ascorbate to dehydroascorbate which in strong acidic medium is hydrolyzed to diketogluconic acid so that hydrozone formation could take place.

Procedure

A sample of 0.2 ml of plasma was pipette into a centrifuge tube and 6.0 ml of 5 percent TCA was added. The contents were mixed thoroughly with a vortex mixer and centrifuged, and 2.0 ml of filtrate was pipette into each of sample control tube. Next, 2 ml of ascorbate standard was added to each standard and standard control tube. Then, one drop of indophenol reagent was added to all the test tubes and mixed well followed by addition of 0.5 ml of DT mix to sample and standard tubes. All the test tubes were incubated for one hour in a water bath at 60°C and cooled in ice water. DT mix (0.5 ml) was pipetted into each sample control tube and standard control tube. While all the tubes remained in ice bath, 2.5 ml of 85% H₂SO₄ was slowly pipetted into all tubes and mixed well. The absorbance of all tubes was read at 505 nm against distilled water.

Estimation vitamin E

Vitamin E was estimated by the method of Kayden et al. (1973) and was based on the principle that vitamin E reduced ferric ions to ferrous ions quantitatively, and these combined with bathophenanthroline to form an orange coloured complex. After adding phosphoric acid to stabilize complex, the colour was read at 536 nm.

Procedure

Volumes of 0.6 ml of plasma, of standard vitamin E and of glass distilled water were pipette into three glass stopped centrifuge tubes marked test, standard and blank, respectively. Then, 0.6 ml of absolute alcohol was added to test and to blank and 0.6 ml of distilled water to standard. The tubes were then stoppered and mixed thoroughly on a vortex mixer. To each tube, 0.6 ml of purified xylene was added and mixed for two minutes on a vortex mixer. Then, the tubes were centrifuged at

\[
\text{Vitamin C (mg/dl)} = \frac{A \text{ of sample} - A \text{ of sample control}}{A \text{ of standard} - A \text{ of standard control}} \times 2
\]
800 g for 5 minutes.

From each centrifuged tube, 0.4 ml of xylene extract was carefully pipetted into fresh appropriate level glass stoppered test tubes containing 0.2 ml of bathophenanthroline reagent and mixed. Then, 0.2 ml ferric chloride reagent was added followed by 0.2 ml of o-phosphoric acid. The contents were mixed thoroughly on a vortex mixer after every addition of reagent. The absorbance of test ($A_t$) and standard ($A_s$) was read at 536 nm against blank.

Plasma vitamin E was estimated according to following equation:

$$\text{Vitamin E (μmol/L) = } \frac{A_t}{A_s} \times \text{X concentration of standard (μmol/L)}$$

solution was diluted with distilled water make the volume to 1 liter. The resulting solution contained 1000 ppm selenium. In case of selenourea, on the basis of molecular weight, 1.558 g was added to 1 liter distilled water so as to obtain 1000 ppm selenium from selenourea. Both the selenium sources were then serially diluted to get various dilutions of selenium: 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 40.0 and 100 μg se/ml. These dilutions were used to get a standard graph.

One ml of each of the above dilutions were placed in a 250 ml conical flask and 5 ml of digestion mixture was added. Condensers (coolers) filled with cold water were kept in the mouth of conical flasks so as to avoid evaporation losses. The flasks were heated at around 160-180°C on a hot plate until dense white fumes started to appear. This took around 5-7 minutes. The flasks were then removed from the hot plate and cooled for 20-25 minutes at room temperature. The contents of the flask were rinsed with 20 ml distilled water, and the bottoms of the condensers were also rinsed. The contents of the flask were thus dissolved and then placed into a separate 50 ml conical flask. To each flask, 2-3 drops of metacresol purple indicator was added, and the solution turned pink. This solution was then brought to pH 7 by adding saturated sodium hydroxide at which the colour became purple. Around 5 ml of saturated NaOH was required in each flask. Now the sample solution was brought to acid pH (2-3) with the help of 90 percent formic acid. To bring the pH to 2.5-2.6 about 1 ml of formic acid was required, resulting in a yellow colored solution. To reduce the oxidative reagents, 4 ml of 40 percent hydroxylamine was added to each sample followed by the addition of 4 ml 0.2 ml EDTA. The solution started turning greenish after the addition of hydroxylamine EDTA, which was added to mark any interfering ions. Again, the pH was checked to ascertain if it was between2-3. If it was lower than 2, it was raised with few drops of liquor ammonia, and if higher, formic acid was used to lower it. For complexion of selenium, 2 ml of 0.5 percent diaminobenzidine solution was used.

Estimation of selenium

The selenium levels in whole blood, hair and tissues were analyzed by the spectrophotometric method described by Cummins et al. (1965) with slight modifications.

Procedure

The standard curve for selenium was prepared by using both sodium selenite and selenourea as the source of selenium. To 80 ml of 40 percent hydrobromic acid around 60 ml distilled water was added to which 2.190 g sodium selenite was mixed. A yellow solution was formed and this was mixed thoroughly on a vortex mixer after every addition of reagent. The solution was then brought to pH 7 by adding saturated sodium hydroxide at which the colour became purple. Around 5 ml of saturated NaOH was required in each flask. Now the sample solution was brought to acid pH (2-3) with the help of 90 percent formic acid. To bring the pH to 2.5-2.6 about 1 ml of formic acid was required, resulting in a yellow colored solution. To reduce the oxidative reagents, 4 ml of 40 percent hydroxylamine was added to each sample followed by the addition of 4 ml 0.2 ml EDTA. The solution started turning greenish after the addition of hydroxylamine EDTA, which was added to mark any interfering ions. Again, the pH was checked to ascertain if it was between2-3. If it was lower than 2, it was raised with few drops of liquor ammonia, and if higher, formic acid was used to lower it. For complexion of selenium, 2 ml of 0.5 percent diaminobenzidine solution was used.
added to each flask as the complex formation takes place only in a pH of 2-3.

The flasks were then incubated in a water bath at 60°C for exactly 20 minutes, after which they were removed and again the pH was adjusted. The pH was brought to 7.0 to 7.5 with the help of liquor ammonia. Around 3-4 ml liquor ammonia was required each time. The solution was then placed in a 125 ml separating funnel, and again the flask was rinsed with few ml of distilled water. Fourmilliliters of toluene was added for selenium extraction. The separatory funnel was vigorously shaken for one minute and then allowed to stand for 2-3 minutes to allow separation of the toluene layer. The lower liquid was discarded, and the toluene was collected in a centrifuge tube. The tubes were centrifuged for few minutes to remove any traces of water. The yellowish coloured toluene layer thus obtained was read for absorbance at 420 nm against clear toluene blank. The absorbance values of different concentrations were obtained and after several replications a standard graph of concentration of selenium vs. absorbance (00) was plotted for both sources of selenium: sodium selenite and selenourea.

Every time a reagent blank was run, as the water in the solutions may also have had selenium, the reading of absorbance of the reagent blank was subtracted from the sample absorbance values to get the actual reading of samples.

**Statistical analysis**

Statistical analysis was done by using completely randomized design (CRD) and randomized block design (RBD) as per the method of Snedecor and Cochran (1968).

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**RESULTS AND DISCUSSION**

**Observations of the animals of Group 1**

**History and symptoms**

The animals in this group had never suffered from mastitis. They were of age group 3-5 years. The mammary glands of these animals revealed normal glandular parenchyma. The animals were mostly recently calved. Colour, consistency, opacity and thickness of milk were normal. There was an absence of any flakes or blood in milk.

**Vitamin C**

Mean vitamin C level in plasma ranged from 1.0795 ± 0.019 to 1.088 ± 0.025 mg/dl (Table 1). There was no significant variation in vitamin C level during the observation period (Figure 1). Chaiyotwittayakun et al. (2002) who states that mean ascorbic acid concentration rapidly increased after the second infusion of vitamin C injection i.e. 650 μmol/l (11.44 mg/dl) and rapidly dropped after wards to below 100 μmol/l (1.76 mg/dl).

**Vitamin E**

The mean value of vitamin E in plasma ranged from 3.983 ± 0.064 to 3.946 ± 0.133 μmol/l (Table 1). There was no significant variation in vitamin E level during the whole observation period (Figure 2). Ndiweni et al. (1991) found that mean plasma vitamin E level above 4 μg/ml i.e. 8.46 μmol/l is regarded as adequate for cattle.

Plasma concentration of greater than 3.5-4 μg/ml of α-tocopherol are considered adequate as evidenced by the relationship between intracellular killing of bacteria by neutrophils and plasma vitamin E concentration (Hogan et al., 1993a).

Vitamin E injected would mitigate the normal peripartum drop in serum α-tocopherol, reduce the impairment of neutrophil function...
and thereby decrease incidence of early lactation clinical mastitis (LeBlanc et al., 2002).

**Selenium**

The mean value of selenium ranged from 0.611 ± 0.059 to 0.62 ± 0.072 μg/ml (table 1). There was no significant variation in selenium levels during whole observation period (Figure 3). Milk neutrophils from cows fed selenium-deficient diets may also be compromised in their capacity to evoke effective responses to microorganisms known to be mastitis pathogens. Neutrophils obtained from cows fed the Se-deficient diet had approximately one-third the microbicidal activity of those from cows fed the Se-supplemented diet. The extra cellular H$_2$O$_2$ concentration was significantly higher in neutrophils harvested from milk of cows fed the Se-deficient diet. This is due to result of reduced catabolism of H$_2$O$_2$ rather than to greater production. Although the lipid structure of the phagolysosome protects the neutrophil from low amounts of H$_2$O$_2$, increased amounts of lipid peroxide is a potential consequence of Se deficiency and can result in damage to cellular and sub-cellular membranes. Catalase and vitamin E are protective agents that may serve to sequester H$_2$O$_2$ and lipid radicals produced by neutrophils. The bovine neutrophil may be particularly susceptible to increased concentration of H$_2$O$_2$ because of the low amounts of cellular catalase. The higher concentration of H$_2$O$_2$, production in Se-deficient neutrophils has been associated with a decrease in microtubule assembly, a decrease in degranulation and damage to the myeloperoxidase systems. Loss of any of these functions would result in less efficient neutrophil bactericidal activity. The peroxides generated also lead to damage to epithelial tissue in which neutrophils have accumulated. In the mammary gland, this damage could lead to irreversible loss of secretory tissue and reduction of milk yield from that gland.

Selenium deficiency in the neutrophil may involve the association of Se with the lipoxygenase pathway of eicosanoid metabolism. High concentrations of hydroperoxides are inhibitory to cyclo-oxygenase and 5-lipoxygenase and Se may protect the cycloxygenase enzyme. A decrease in leukotriene B$_4$, a product of lipoxygenase pathway occur in neutrophils obtained from Se-deficient cows. Leukotriene B$_4$ enhances chemotaxis, recognition and degranulation of neutrophils. A decrease in leukotriene B$_4$ as a result of Se deficiency may result in a partial loss of these functions. A decrease in degranulation may result in a decreased killing capacity of neutrophils.

**Observation of animals of Group 2**

**History and symptoms**

The animals were in their 3rd to 5th lactation and recently calved. More hind quarters were infected than fore quarters (60% vs. 40%). This might be due to more exposure of hind quarters to floor urine and dung and higher milk content in hind quarters than in fore quarters. This finding is similar to finding of Rao and Naidu (1969) and Kapur and Singh (1978). Incidence was highest during first three months of lactation i.e. 60%. Similar observations have been recorded by Kapur and Singh (1978). The presence of pain response (purposeful lifting and kicking of the adjacent hind limb) during palpation of the mammary gland was noted (Maunsell et al., 1998). There was a presence of flakes in the milk of most of the animals. Out of six animals, blood was present in the milk of two animals. The consistency of milk was watery in three animals out of the six.
Vitamin C

Pre-treatment and post-treatment value of vitamin C ranged from 0.765 ± 0.128 to 0.924 ± 0.088 mg/dl and 1.0795 ± 0.019 to 1.088 ± 0.025 mg/dl, respectively (Table 2), i.e. a 28% increase in level after treatment. There was a significant difference (5%) between pre-treatment and post treatment levels of vitamin C in the animals of this group (Figure 2). When compared with vit C level of animals of Group 1, there was a significant difference (5%). Four out of the six animals were cured symptomatically, but culturally they were positive for mastitis. Two animals did not recover and antibiotics based on culture sensitivity testing were prescribed for them. In one animal, mammary gland became fibrosed. On the basis of the case recovery rate of 80%, it can be concluded that injredoxin can be used as an adjunct therapy in treatment of recurrent cases of mastitis.

Ascorbate functions as antioxidants by neutralizing the peroxidase and other free radicals responsible for tissue injury thus protecting cells of body. Beside its anti-oxidant properties, ascorbate also has a pre-oxidant role to activate myeloperoxidase inside the neutrophils and thus enhancing the immune response.

Further ascorbate, a cofactor in the hydroxylation reaction of proline and lysine, is also involved in the maintenance of natural barriers. Thus a depression in udder defense due to high production and lower ascorbic acid concentration may initiate multiplication of mastitogens (Singh and Pachauri, 2003).

Observations of the animals of Group 3

History and symptoms

The animals in this group were in their first to third lactation, four to sixth month of calving and recently calved. All animals had previously been treated with lemox (ampicillin and cloxacillin) but there was reoccurrence of mastitis. The affected quarters were swollen, hot, oedematous and painful to touch in starting. Then, after treatment, four animals out of six were cured and returned to normal condition. The inflammation subsided and the animal became clinically normal. One animal out of the six was not cured. In one animal, the mammary gland became fibrosed.

Vitamin E

Pre-treatment and post-treatment value values of vitamin E ranged from 2.58 ± 0.24 to 3.006 ± 0.434 μmol/l and 3.983 ± 0.064 to 3.946 ± 0.133 μmol/l, respectively, showing a significant difference i.e. 41% increase in level (Table 3). While comparing it with vitamin E level of animals of Group 1, it showed significant difference (5%) (Figure 2). Four out of six animals were cured symptomatically, but culturally they were positive for mastitis. Two animals did not recover and antibiotics based on culture sensitivity testing were prescribed for them. The case recovery rate of 80% suggests that inj vetade can be used as an adjunct therapy in the treatment of mastitis.

Plasma concentrations of α-tocopherol are low during peripartum period (Goff and Stabel 1990; Weiss et al., 1990a; Weiss et al., 1994; Hogan et al., 1997).

The periparturient period is associated with decreased activity of PMN and with increased susceptibility to mastitis. An increase in vitamin E levels after parturition could improve the animal immune responses and thus leads to lower incidence of mastitis (Ndiweniet al., 1991).

Vitamin E is an integral component of all lipid membranes and serves to protect lipid membranes from attack by reactive oxygen species. Polyunsaturated fatty acids of membranes are
Table 1. Mean values of vitamin C and vitamin E of animals of Group 1.

<table>
<thead>
<tr>
<th>Days of observation</th>
<th>Vitamin C (mg/dl)</th>
<th>Vitamin E (μmol/L)</th>
<th>Selenium (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>1.0795 ± 0.019</td>
<td>3.983 ± 0.064</td>
<td>0.611 ± 0.059</td>
</tr>
<tr>
<td>7 day</td>
<td>1.088 ± 0.025</td>
<td>3.946 ± 0.133</td>
<td>0.62 ± 0.072</td>
</tr>
<tr>
<td>14 day</td>
<td>1.080 ± 0.014</td>
<td>3.941 ± 0.216</td>
<td>0.60 ± 0.05</td>
</tr>
<tr>
<td>21 day</td>
<td>1.081 ± 0.0134</td>
<td>4.958 ± 0.194</td>
<td>0.61 ± 0.05</td>
</tr>
</tbody>
</table>

Table 2. Mean values of vitamin C of Group 2.

<table>
<thead>
<tr>
<th>Days of observation</th>
<th>Vitamin C (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>0.924 ± 0.088*</td>
</tr>
<tr>
<td>7/0 day*</td>
<td>0.765 ± 0.128x</td>
</tr>
<tr>
<td>14/7 day</td>
<td>1.088 ± 0.025x</td>
</tr>
<tr>
<td>21/14 day</td>
<td>1.0795 ± 0.019*</td>
</tr>
</tbody>
</table>

* indicates day of treatment with injRedoxin.

x indicates significant difference at 5% confidence interval.

Table 3. Mean values of vitamin E and Selenium of Group 3.

<table>
<thead>
<tr>
<th>Days of observation</th>
<th>Vitamin E (μmol/L)</th>
<th>Selenium (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>3.445 ± 0.1899*</td>
<td>0.245 ± 0.068*</td>
</tr>
<tr>
<td>7/0 day*</td>
<td>3.303 ± 0.1576x</td>
<td>0.19 ± 0.062x</td>
</tr>
<tr>
<td>14/7 day</td>
<td>6.73 ± 0.1474x</td>
<td>0.695 ± 0.031x</td>
</tr>
<tr>
<td>21/14 day</td>
<td>4.265 ± 0.1157x</td>
<td>0.690 ± 0.025x</td>
</tr>
</tbody>
</table>

* indicates day of treatment with injVetade.

x indicates significant difference at 5% confidence interval.
Figure 1. Comparison of vitamin C levels of Groups 1 and 2.

Figure 2. Comparison of vitamin E levels of Groups 1 and 3.

Figure 3. Comparison of selenium levels of Groups 1 and 3.
particular vulnerable to attack by reactive oxygen species and ROS can initiate a chain reaction of lipid destruction that destroys the membrane of the cell. Vitamin E can quench peroxidation reactions in membranes.

The arachidonic acid metabolism is altered in animals deficient in vitamin E, selenium or both (Atroshi et al., 1989; Aziz and Klesius, 1986). Vitamin E may function to control peroxidation of arachidonic acid or its unstable metabolites. The arachidonic acid metabolites are important for PMN function and the amplification of inflammatory response following pathogen invasion of tissues including the mammary gland (Aziz et al., 1984). The speed with which PMN can be mobilized following pathogen invasion and the efficiency of intracellular kill are events of critical importance to protection of the mammary gland from infection (Smith et al., 1997). Vitamin E and Se play essential roles in these events and dietary deficiencies of either leads to impaired PMN function and increased incidence of intramammary infection in dairy cows (Hogan et al., 1993a).

Selenium

Pre-treatment and post-treatment value values of selenium ranged from 0.19 ± 0.062 to 0.245 ± 0.068 μg/ml and 0.690 ± 0.025 to 0.695 ± 0.031 μg/ml, respectively, showing a 2.18% increase in level (Table 3). There was significant difference (5%) between pre- and post-treatment levels of selenium.

Mukherjee (2008) also found that treatment of mastitis in buffaloes with vitamin E and selenium deficiencies. Mammary gland infections and clinical mastitis were not affected by treatment, but milk production was increased by treatment; milk production was also increased by feeding high amounts of vitamin A (Weiss, 1998).

Milk neutrophils from Se-deficient cows had impaired bactericidal activity compared to Se-supplemented cows. This indicates an important role of Se in mammary resistance to infection (Grasso et al., 1990).

A dose of 0.1 mg Se/kg of body weight at 21 days before calving had no effect on incidence of clinical mastitis. However cows supplemented with both vitamin E and Se had shorter duration of clinical signs than cows supplemented with either micronutrient alone (Smith et al., 1997).

Vitamin D also is involved with immune function (Reinhardt and Hustmyer 1987). Increased lymphocyte proliferation was observed when Jersey cows were infused subcutaneously with 50 μg of 1, 25-dihydroxy vitamin D/day for 7 days (Hustmyer et al., 1994).

CONCLUSION

We can forecast the incidence of mastitis in a particular herd by estimating vitamin E and vitamin C levels in plasma of animals. Further, antioxidant treatment may have cured the epithelium of teat canal and mammary gland of mastitis affected animals. Integrity of epithelium has been maintained as no cases of mastitis were recorded after treatment.

REFERENCES


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…used liquid nitrogen vapour freezing technique from Verma et al. (1975)
…and buffaloes (Singh et al., 1983; Shah et al., 1987; Misra, 1996; Pant et al., 2002)

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