EFFECT OF CLITORAL STIMULATION AFTER ARTIFICIAL INSEMINATION ON CONCEPTION RATE IN BUFFALOES

Thesis

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Certificate

Certified that the research work embodied in this thesis entitled **"Effect of clitoral stimulation after artificial insemination on conception rate in buffaloes"** *submitted by* **Dr. Vipin Maurya, Roll No. 5307**, *for the award of* **Master of Veterinary Science** *degree in* **Livestock Production and Management** *at* **Indian Veterinary Research Institute, Izatnagar**, *is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that **Dr. Vipin Maurya, Roll No. 5307**, *has worked for more than 21 months in the institute and has put in more than 150 days attendance under me from the date of registration for the Master of Veterinary Science degree in this Deemed University, as required under the relevant ordinance.*

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We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by him for the award of **Master of Veterinary Science** *degree of this institute.*

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या वीणावरदण्डमण्डितकरा या श्वेतपद्मासना ।
या ब्रह्माच्युतशंकरप्रभृतिभिर्देवः सदा पूजिता
सा मां पातु सरस्वति भगवती निःशेषजाड्यापहा ॥

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ABBREVIATIONS

Abbreviation		Full name
%	:	percentage
µg/ml	:	microgram per mililitre
AI	:	Artificial Insemination
ANOVA	:	Analysis of variance
C.I	:	Confidence Interval
CL	:	corpus luteum
CR	:	conception rate
DF	:	Degree of freedom
E_2	:	Estradiol 17β
ELISA	:	Enzyme linked immunosorbent assay
FSH	:	Follicle stimulating hormone
GDP	:	Gross Domestic Product
GnRH	:	Gonadotrophin releasing Hormone
h	:	hour
Hcg	:	Human chorionic Gonadotrophin
HCL	:	Hydrochloric acid
i.e	:	that is
LH	:	Luteinizing Hormone
ng/ml:	:	nano gram per mililitre
O.D.	:	Optical Density
$P<0.01$	:	Significant at 1% level
$P<0.05$	:	Significant at 5% level
P_4	:	Progesterone
PR	:	Pulse rate
RIA	:	Radioimmunoassay
rpm	:	Rotation per minute
RR	:	Respiration rate
THI	:	Temperature humidity index
TMB	:	3,3',5,5'-Tetramethylbenzidine
β	:	beta

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Introduction

1 INTRODUCTION

The food demand is continuously increasing and expected to be doubled by the year 2050 due to population explosion while agricultural productivity is declining largely due to shrinking of resources particularly cultivable land. Role of livestock to ensure human nutritional security is of paramount importance under these circumstances. The livestock sector has emerged as one of the key components of agricultural growth in developing countries including India and plays a vital role in the socio-economic upliftment through employment generation. It provides not only quality animal protein in the human diet in the form of milk, meat, egg etc. but also a source of draught power, manure, hides and other products. Over the last three decades, livestock sector has consistently accounted for over 4 % of the country's GDP, while its share in the GDP from agricultural sector steadily increased from 14 to 25%, Milk is the major contributor to the GDP from livestock sector (www.icar.org.in).

Buffalo is one of the most important dairy animal concentrated largely in tropical and sub-tropical countries. Buffalo husbandry is an important source of income and means of employment for more than 70 million farmers in India. 55.5% of the total milk produced in India comes from buffaloes followed by cattle (40%) and goat (4.5%) respectively (Dairy India, 2007). With such high contribution of buffalo, today India is the largest milk producer in the world producing about 121 million metric tons per annum (NDDB Annual report 2011-12). Considering its importance and production potential, buffalo has been termed as "The Black Gold" (Acharya and Bhat,1988). They are the mainstay of dairy industry, especially in Asia and form the rural world after cattle. India is the largest exporter of buffalo meat as well. Good feed conversion efficiency and relatively low maintenance requirements with higher fat percentage in milk are attributes that make buffalo ideal in low input cost systems (Zicarelli, 1994; Paul et al.,2002). This thrifty, versatile, adaptable and productive domestic animal has drawn national and international attention in the last few decades (Cockrill, 1980). The enhanced interest in the species is evident by the popularization of buffalo farming in Mediterranean area to Latin America and in Central/Northern Europe as well (Barile, 2005). The species is disturbed over more than 40 countries of the world (Gordon, 1996) and the population has increased from 159 million (FAOSTAT, 1997) to 199 million (FAOSTAT, 2013). Preference of Indian farmers is now changing from cattle to buffalo at very faster rate which is evident from 7.99% growth rate and more than 55 per cent of the milk now flows from the udders of buffaloes although buffalo population is less than half (42.4%) of total cattle population (19^{th} Livestock census, 2012).They have immense agricultural importance by virtue of their high production potential through meat and milk for mankind besides being a source of sustenance to poor and marginal farmers as well as landless labourers in the developing world (Gupta and Das, 1994).

Low reproductive efficiency in buffalo remains a major economic problem globally and its incidence is higher in India (Kumar et al 2009). Reproductive efficiency is the primary factor affecting productivity and is hampered in buffalo by several inherent problems including delayed sexual maturity, poor estrus expression, distinct seasonal reproductive patterns, and prolonged intercalving intervals (Madan and Raina, 1984; Agarwal & Tomar, 2003). Although buffaloes are polyoestrus, their reproductive efficiency shows wide variation throughout the year. Buffalo cows exhibit a distinct seasonal change in displaying oestrus, conception rate and calving rate. The age at first oestrus of heifers varies between breeds from 13–33 months but mating at the first oestrus is often infertile and usually deferred until they are 3 years old. The productivity of buffaloes remains low largely due to poor management of health, nutrition (Bal Krishnan and Bakagopal, 1994) and breeding (Rane et al., 2003). Anestrus, silent ovulation and repeat breeding are the major reproductive disorders in buffaloes (Goley and Kadu, 1995). Low conception rate remains the biggest problems of the dairy industry. In spite of its large contribution to total milk and meat production of the country, little efforts have been made to improve their reproductive efficiency and conception rates.

Conception Rate (CR) in buffalo with natural service, chilled semen and frozen semen have been reported as more than 60%, 35-60% and 25-45 % respectively in organized herd.(Jainudeen and Hafez., 1993; Agarwal and Shanker., 1994). Under field condition CR through Chilled and Frozen semen has been reported very low (25-37.3%) (Agarwal and Purbey.,1983; Tailor et al., 1990), which could be attributed to several factors viz. inseminator`s skill, quality of semen, time of breeding, cold chain maintenance, poor heat detection, health status of the animal, etc. Inspite of the complete understating of these different factors that influence the success of technique, CR obtained from artificial insemination (A.I) is relatively low compared to natural breeding. A critical investigation of the physiological effects of management of females at insemination time may help to improve reproductive efficiency.

The mean duration of estrus in buffalo is 21 hrs (range 17-24 hrs). Ovulation occurs 32 hrs after the onset of heat (Hafez, 2000) and between 24 and 30 h after the onset of LH surge (Seren et al., 1995; Porto-Filho et al., 1999). The interval between the onset of oestrus and the LH surge has been reported to be 8 hrs by Kumar et al (1991). Duration of LH Surge is about 6-9 hrs (Yüksel M et al., 2011). The hypophysial hormone LH plays an important role in ovulation and luteinization in females. The control of ovulation is brought about by the interactions between the pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Findley et al.,1996). The pulsatile LH secretion is used as an indirect measure of hypothalamic GnRH secretion (Clark IJ 2002). Positive feedback of estradiol on luteinizing hormone (LH) secretion have been observed (Land et al., 1976; Mori et al., 1987) and hence estradiol has positive feedback for initiating ovulation.

Endocrinogical causes for poor conception through A.I. may be ovarian dysfunction, hormonal imbalance and delayed ovulation. Kavani and Kodagali (1984) reported 16.6% delayed ovulation occurring after 48 hrs of standing heat in buffalo which is higher in buffaloes as compared to 7.9% in cattle (Hernández-Cerón et al, 1993). Ovulation in buffaloes occurs generally between 12-24 hrs after the end of estrus (Danell,

1987) but sometimes it takes longer time. Ovulations occurring beyond 24 hrs after end of heat are considered as delayed ovulations and when the animal fails to ovulate, the condition is anovulation. The underlying physiology of delayed ovulation and anovulation may due to insufficient levels of LH or delayed L.H surge. This might be due to inadequate progesterone priming of hypothalamus or the presence of suprabasal progesterone concentrations during oestrus (Wiltbank et al., 2002) which has an inhibitory effect on the positive feedback of high oestradiol concentrations on the hypothalamus, resulting in inhibition of L.H surge (Stock and Fortune, 1993; Sirois and Fortune, 1990; Savio et al., 1993b; Roche et al., 1999; Noble et al., 2000).There are many techniques to overcome this problem: mostly used is hormonal therapy that includes hCG or LH preparations (1500 to 3000 IU I/V) at the time of AI or GnRH (Receptal 10 ug or Fertagyl 500 mcg) I/M at the time of A.I (Stevenson et al, 1990; Tanabe et al., 1994). GnRH evokes the release of L.H and hence L.H Surge followed by ovulation (Lee et al 1981). Disadvantage of this therapy is that some of these hormones are not easily available, inconsistent effects, may have side-effects and are expensive.

Managemental aspect and non hormonal approach may be explored in buffalo species to overcome this hurdle and C.R may be increased, simply by manual stimulation of clitoris after A.I; which has been demonstrated successfully by Capitan et al (1992) in Philippine swamp buffalo (Carabaos) and several researchers in cattle but so far no study along this line has been done in Indian riverine buffaloes.

Robertson and Rahka (1965) reported that chlorpromazine, a neural blocking agent, given within 2 hr after the onset of estrus blocked ovulation in sheep. VanDemark and Hays (1952) found an increase in uterine motility in cows exposed to the presence of the bull, nuzzling by the bull, noncopulatory mounting and mounting with copulation. Hays and VanDemark (1953a) documented that massage of the vulva and cervix and natural mating produced an increase in intramammary pressure. The same workers (1953b) found that oxytocin caused increased uterine contractions at all stages of the estrual cycle. These data indicate a neural involvement with pituitary release of L.H, oxytocin and other hormones from stimulation of the reproductive tract at mating or artificial stimulus i.e. Various neural pathways exists between the reproductive system and hypothalamic-pituitary axis (neuro-endocrine mechanism). One of the ways to provide such neural stimuli for endocrine response is through clitoris at ventral commissure of the vestibule which has the same embryonic origin like penis. It is composed of erectile tissue covered by stratified squamous epithelium, and it is well-supplied with sensory nerve endings. The massaging of the clitoris in cows enlarges the cervical lumen and allows easier passage (Pointner, 1986). It also stimulates the release of oxytocin causing increased contractions of the uterus toward the oviduct (Coyan and Tekeli, 1996). Stimulation of the reproductive tract as clitoral massage for 10 sec following A.I. hastened the L.H surge, hence ovulation in cattle (Randel et al., 1973).

Clitoral massage positively affects the release of certain protein hormones, such as oxytocin and LH (Randel et al., 1975), which is essential for ovulation. Several researchers found positive effect of clitoral stimulation on CR and reported significantly higher pregnancy rates in treated group as compared to control in cattle. (Glauber, 1989; Segura and Rodriguez., 1994; Singh et al., 2001; Kutty CI; 2006). However, Randel et al., (1975), Short et al.,(1979) and Lunstra et al., (1983) have reported that clitoral stimulation increased the first service pregnancy rate significantly in cows unlike heifers. In Philippine swamp buffalo (Carabaos), Capitan et al (1992) found a significant increase in conception rate both in heifers and parous buffaloes after clitoral massage of 30 seconds. The best positive effects of clitoral massage appear in the second half of the oestrus (Coyan and Tekeli, 1996).

It appears from the facts that clitoral stimulation affects ovulation time and pituitary release of oxytocin and L.H in the cow. These factors may directly or indirectly affect conception rate. Moreover clitoral massage is a managerial practice which may simulate the natural service and in general conception from natural service is more than that of A.I.(Jainudeen and Hafez 1993; Agarwal and Shanker 1994) therefore clitoral stimulation is likely to have an effect on conception rate.

Strong maternal instinct makes weaning difficult in buffaloes at an early age and if calf dies, milk production drops drastically (www.agritech.tnau.ac.in). This indicates that buffalo is more sensitive to neuro-endocrine stimulus and hence clitoral stimulation may induce general excitability which could be reflected by physiological responses in terms of pulse and respiration rates.

To the best of our knowledge no scientific report is available regarding effects of clitoral stimulation at A.I. on the conception rates in riverine buffalo cows and influence on reproductive hormone profiles. Therefore, the present study has been designed with the following objectives:

1. To study the effect of clitoral stimulation after A.I. on reproductive hormone profiles (luteinizing hormone, estradiol and progesterone) and conception rate in buffaloes.

Review of Literature

2 REVIEW OF LITERATURE

In tropical countries like India, the domestic buffalo rearing is an indispensable livestock activity to millions of smallholding farmers. In India buffalo population is less than 40% of the total bovine population, but contributes for more than half (56%) of the total milk production in the country (Ingawale and Dhoble, 2004). India's exports of animal products was Rs. 32288.57 crores in 2013-14, which include the major products like carabeef (Rs. 26,457.79 crores) followed by dairy products (Rs. 3,318.53 crores). The demand for Indian buffalo meat in international market has sparked a sudden increase in the meat exports with a contribution of over 86%. The product registered 27% growth in export during the financial year 2012-13 as compared to the same period of last year (source : APEDA).

However, Reproductive efficiency is the primary factor affecting productivity and is hampered in female buffalo by inherent problem viz. late maturity, poor estrus expression in summer, distinct seasonal reproductive patterns and prolonged intercalving intervals (Madan and Raina, 1984; Agarwal & Tomar, 2003). Indian buffaloes are considered difficult breeders because of their greater susceptibility to environmental stress which causes anoestrus and repeat breeding, besides delayed age at first calving and higher incidence of silent ovulation (Usmani et al., 1985; Madan, 1988). Various aberrations of oestrus manifestations are experienced in animals bred continuously through AI, which include long duration of heat lasting for 2 or 3 days (Kutty and Ramachandran, 2000; Nasir, 2003) instead of 12-18 hr normally expected.

Prolongation of heat is attributable to insufficient luteinization of granulosa cells and delayed ovulation leading to failure of service unless AI is done more than once during the same heat period (Arthur et al., 1989; Duchens et al., 1995; Gatius et al., 2002). Higher incidence of delayed ovulation (Kavani and Kodagali, 1984) and poor conception rate upon AI, affect productivity and cause economic losses to buffalo producers (Agarwal and Purbey.,1983; Tailor et al., 1990).

Robertson and Rahka (1965) reported that chlorpromazine, a neural blocking agent, given within 2 hr after the onset of estrus blocked ovulation in sheep, suggesting that a neural connection between the release of gonadotropin and ovulation exists in sheep. VanDemark and Hays (1952) found an increase in uterine motility in cows exposed to the presence of the bull, nuzzling by the bull, noncopulatory mounting and mounting with copulation, possibly resulting in improved sperm transport (Karaca et al., 2001). Cooper et al. (1985) reported that clitoral massage applied at time of AI caused an immediate uterine contraction in cows, and this response was neurally rather than hormonally mediated. Hays and VanDemark (1953a) found that massage of the vulva and cervix and natural mating produced an increase in intramammary pressure. The same workers (1953b) found that oxytocin caused increased uterine activity at all stages of the estrual cycle. Nikolakopoulos et al. (2000) have reported that events around mating including stimulation of the genital tract and uterine distension often caused an increase in circulating concentrations of oxytocin. It has been reported that estrogens and testosterone treatments accelerate vaginal and clitoral blood flow (Min et al., 2002; Park et al., 2001) and increase vaginal lubrication (Park et al., 2001).

2.2 Clitoral Stimulation and Conception Rate

Clitoris and vestibule are the two segments of the female reproductive tract provided with sensory innervations (Roberts, 1971; Bearden and Fuquay, 1980). Anatomical features of clitoris gives indication of its biological role in conveying biological signals to the female system regarding mating process so as to initiate hormonal mechanisms leading to ovulation and cessation of heat sign (Nikolakopoulos et al., 2000). Clitoris which has the same embryonic origin like penis is located at ventral commissure of the vulva. It is composed of erectile tissue covered by stratified squamous epithelium, and it is well-supplied with sensory nerve endings. Various neural pathways exists between the reproductive system and hypothalamic-pituitary axis (Frandson, 1975; Hafez et al., 2000).

Randel et al. (1973) reported that stimulation of the reproductive tract by 10 sec of manual clitoral massage following A.I. hastened the L.H surge and ovulation in the cow. Chance of stimulating clitoris and vestibule appears to be minimal in the process of AI and the consequent lack of sensory stimulation in artificially bred animals can be thought to affect the biological signals for ovulation (Randel et al., 1975) leading to persistence of heat signs, delayed ovulation and low conception rate. Manual clitoral stimulation has been shown to shorten the interval from estrus to ovulation and increase pregnancy rates in lactating beef cows (Randel et al., 1975; Stupnicki, 1975) unlike heifers (Randel et al., 1975; Short et al., 1979). The exact cause of this effect is not known, but it may involve the effect that stimulation has on time of ovulation, sperm transport mechanisms (Van Demark and Hays, 1952) or both. Neither is it known why stimulation failed to increase pregnancy rates in heifers. It is possible that the 10 sec of stimulation used previously was too long for heifers as some of them seemed to become uncomfortable. On the contrary, Cooper and Foote (1986) reported that clitoral massage applied for 5 seconds after insemination did not alter the pregnancy rate (control 74.0%, clitoral massage 74.3%) in cows, although it increased in heifers (control 70.6%, clitoral massage 81.9%). Segura and Rodriguez (1994) reported that clitoral stimulation significantly increased pregnancy rates (57% versus 45% for treated and control groups, respectively) in Zebu-cross-bred heifers. Arbeiter et al. (1985) reported that the pregnancy rate was found to be higher in heifers to which clitoral massage was applied (80%) than that of those to which it was not (40%). Glauber (1989) also found that conception rates were higher in cows on which clitoral massage was carried out for 10 seconds (65.08%) than in controls (51.43%). Lunstra et al. (1983) reported that clitoral stimulation increased the first service pregnancy rate in cows (74 ± 3% versus 59 ± 3%, $P<0.05$), but not in heifers (53 ± 5% versus 57 ± 5%, $P>0.10$), and that clitoral stimulation improved pregnancy rates more effectively in 3- to 4-year-old cows, than in both 2- year-old and 5-year-old, or even older, animals. Capitan et al (1992) in Philippine swamp buffalo (carabaos) found a significant increase in conception rate both in heifers and parous buffaloes after clitoral massage of 30 seconds. Although Karaca et al., (2001) documented that the pregnancy rates of cows with clitoral massage and non-clitoral massage were found to be 74.29% and 67.65%, respectively, though these differences were non-significant. However, Rodriguez et al. (1980) showed that pregnancy rate was higher in animals not receiving clitoral massage than in those receiving it.

The massaging of the clitoris in cows enlarges the cervix lumen and allows easier passage (Pointner, 1986), stimulates the release of oxytocin, and this hormone increases the contractions of the uterus toward the oviduct (Coyan and Tekeli, 1996) on the contrary, clitoral massage causes an increase of uterine contractions without enhancing the release of oxytocin (Cooper et al., 1985; Cooper and Foote, 1986). The best positive effects of clitoral massage appear in the second half of the oestrus (Coyan and Tekeli, 1996). However, Kirsch et al. (1985) suggested that stimulation of the pelvic region during both natural mating and A.I.does not enhance the release of LH in gilts.

Custer et al. (1990) and Lunstra et al. (1982) have reported that increased biostimulation of the female during natural mating results in higher pregnancy rate. Mating can modulate the pre-ovulatory surge of LH by prolonging the duration of LH release rather than by increasing plasma concentrations. Mating affects the time of ovulation in spontaneously ovulating species such, as sheep. In sows, natural mating affects ovulation by shortening the interval from onset of estrus to ovulation and by reducing the interval from the first to last ovulation. Naturally mated sows have higher concentrations of plasma LH immediately after mating (Hafez, 2000).

Sexual stimulation of cows is an important factor in the success rate of A.I (Stupnicki, 1975). It has been reported that the exposure of bulls to postpartum cows reduced the interval from calving to the resumption of luteal activity in primiparous (Gifford et al., 1989; Custer et al., 1990; Fernandez et al., 1993) as well as multiparous cows (Zalesky et al., 1984; Alberio et al., 1987; Naasz and Miller, 1990; Burns and Spitzer, 1992). Presence of male before the breeding season stimulated onset of ovarian activity in seasonally anestrus ewes (Knight and Peterson, 1978) goats (Shelton, 1960) and suckled sows (Rowlinson et al., 1975). Continuous bull exposure of buffaloes during later postpartum perod (40 days post-calving) accelerates postpartum resumption of ovarian cyclic activity, reduces incidence of silent ovulation and enhances first service conception rate (Gokuldas et al, 2010). Fernandez et al. (1996) observed that bull exposure caused an increase in the pulse frequency of L.H from the anterior pituitary.

Clitoral stimulation at the time of A.I have been reported as an effective means of increasing pregnancy rate in dairy cows (Singh et al., 2001; Lunstra et al., 1983). Clitoral massage shortens the estrus duration in crossbred cattle herds with prolonged oestrus, in terms of number of days of showing the heat signs. This in turn reduces the necessity for double AI and improves the conception rate of single AI, lowering number of services preconception (Kutty, 2006). Arbeiter et al. (1985) observed that a more rapid follicle growth in clitoral massage group and ovulation occured earlier compared with the control group.

It is known that resumption of ovarian cycling activity is characterized by the release of GnRH by the hypothalamus in low amplitude high frequency temporal release pattern (Wright et al., 1992). As a result, the anterior pituitary releases low amplitude, high frequency pulses of LH which in turn stimulates ovulation and resumption ovarian cycling activity (Rhodes et al., 2003; Chagas et al., 2006). Unlike in mice (Singh, 2001) and goats (Iwata et al., 2000) aspects of biostimulation such as the behavioral and pheromonal signal and its translation into a hormonal response remain unclear in cattle and buffaloes.

The rapid transport of live or dead sperm to the upper oviduct within a matter of minutes infers the importance of smooth muscle contractions in this situation. In the natural breeding, sperm are deposited during estrus at least 10 to 12 hrs before ovulation, and the rate of sperm transport may be a critical factor for determining the conception rate. In the case of A.I., particularly when this forms a part of an estrous synchronization and/or gonadotropin treatment program, the rate and efficiency of sperm transport to the site of fertilization are of fundamental importance (Hafez, 2000). Immediately after insemination, sperm penetrate the micelle of the cervical mucus where some are quickly transported through the cervical canal. This phase takes 2 to10 minutes and may be facilitated by sperm motility as well as increased contractile activity of the myometrium and mesosalpinx during courtship and coitus. Some sperm reach the internal os of the cervix within 1.5 to 3 minutes after insemination. Thus, some sperm can reach the site of fertilization rapidly (Ptaszynska, 2006). Whether the first sperm entering the oviduct participate in fertilization of the ovum is not known, it has been proposed that fertilization occurs only when a minimal number of sperm reach the site of fertilization. The stimulating of the vagina at coitus causes a reflex release of oxytocin, which in turn lead to contraction of the genital tract smooth muscles and increases the rate of sperm transport. On the other hand, clitoral stimulation at the time of A.I.causes oxytocin release from the posterior pituitary gland, which has positive effect on the sperm transported into female genital tract (Bozkurt et al., 2007). Oxytocin was used to increase CR by improving the sperm transport in the female reproductive tract of several species (Hays et al., 1958; Hawk 1987; Sayre and Lewis 1997; King et al., 2004; Yildiz, 2005).These results indicate a neural involvement with pituitary release of oxytocin from stimulation of the reproductive tract at the time of breeding.

It appears from the above reports that mechanical stimulation of reproductive tract hastens release of oxytocin, L.H surge and ovulation in cows. Many attempts have been made to increase fertility in lactating dairy cows. Gonadotropin-releasing hormone (GnRH) and its analogues administered at the time of AI are the most common treatments in management programmes for herds, recommended by several researchers (Stevenson et al., 1984; Chenault, 1990; Stevenson et al., 1990; Morgan and Lean, 1993). Improvement in the conception following GnRH treatment has been attributed to the prevention of an ovulation failure or a reduced variation in the interval between the onset of oestrus and ovulation by means of the induced preovulatory LH surge (Mee et al., 1993; Kaim et al., 2003). However the results are controversial after GnRH treatment of lactating cows. Some researchers found out that CR was improved (Kaim et al., 2003; Lee et al., 1983; Nakao et al., 1983; Lopez- Gatius et al., 2006) while others reported no effect on conception (Stevenson et al., 1984; Lee et al., 1985; Chenault, 1990; Mee et al., 1990; Perry and Perry, 2009).

Various attempts made to regulate postpartum reproduction through use of hormones have so far failed to be consistently effective and besides, some of these preparations may have side-effects, are not easily available, unsustainable or too expensive for smallholder farmers. For harnessing the reproductive potential of buffaloes, there is a need to develop rational management strategies that will curtail reproductive problems, without reducing milk yield or calf growth rates, and without significant increases in production costs. In particular, low CR following AI in buffalo needs to be targeted for exploiting the fullest production potential of this versatile species. Clitoral stimulation is an inexpensive management tool. While there is reasonable evidence to support the effect of clitoral stimulation increasing conception in cattle, there exists a paucity of information concerning this application in buffaloes.

2.3 Luteinizing hormone (LH)

The hypothalamus is responsible for control of release of gonadotrophins from anterior pituitary by the action of specific releasing and inhibitory substances. These are secreted from hypothalamic neurons and are carried by hypothalamic-hypophysial portal system. Of the neuro-endocrine peptides that play critical role in the control of reproduction in vertebrates, the decapeptide GnRH also termed L.H releasing hormone (LHRH) for its preferential action on LH secretion, is the key molecule that acts on pituitary gonadotropes to stimulate the secretion of gonadotropins viz., LH and follicle stimulating hormone (FSH) (Kaltenbach et al, 1974; Schams et al, 1974) and these hormones in turn act on the ovary to stimulate its steroidogenesis and gametogenesis. Generally, the ovarian steroids act on the hypothalamus to regulate GnRH secretion. There is good evidence that in domestic species the secretion of FSH and LH is controlled by two functionally separate, but superimposable, systems. These are the episodic/tonic system, which is responsible for the continuous basal secretion of gonadotrophin and stimulates the growth of both germinal and endocrine components of the ovary, and the surge system, which controls the short-lived massive secretion of gonadotrophin, particularly LH, responsible for ovulation. Not only does the anterior pituitary have a direct effect upon ovarian functions by stimulating folliculogenesis, follicular maturation, ovulation and corpus luteum formation, but the ovary has an effect upon the hypothalamus and anterior pituitary. This is mediated by estradiol, produced by the maturing follicles and progesterone by the corpus luteum. The episodic/tonic hypothalamic release centre is influenced by the negative-feedback effect of estradiol and progesterone and surge centre is regulated by positive-feedback effect of estradiol. Tonic release of gonadotrophins, especially LH, does not occur at a steady rate but in a pulsatile fashion in response to a similar release of GnRH from the hypothalamus. The negative-feedback of progesterone is mediated via a reduction in pulse frequency of gonadotrophns release, while estradiol exerts its effect via a reduced pulse amplitude. It is possible that the negative-feedback of progesterone on LH release may be mediated via opioids (Brooks et al., 1986). LH induces ovulation and luteinisation of the granulosa and thecal cells leading to development of corpus luteum. It also appears to be the principal luteotrophic hormone in the bovines (Peters and Lamming, 1983).

The LH structure is similar to that of the other glycoprotein hormones, FSH, thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG). Pre-ovulatory peak of LH is responsible for the follicular wall rupture and ovulation (Maurel *et al*., 1995). The peripheral LH remains at basal levels throughout the estrous cycle till the day of estrus when a pre-ovulatory LH surge occurs (Rahe et al., 1980). Whereas the baseline values is around 0.72-3.0 ng/ml during a major part of the estruos cycle in cattle, peak LH values of 20-40 ng/ml have been observed on the day of oestrus (Arora et al., 1982; Kanai et al., 1984; Avenell et al., 1985)

The interval from E2 peak to LH peak has been reported to be 14-15 h and the length of the LH peak in the Mediterranean Italian buffalo has been estimated to be 6-12 hrs (Seren et al., 1994; Maurel et al., 1995). The interval between the onset of oestrus and the LH surge has been reported to be 8 hrs (Kumar et al 1991). Kanai and Shimizu (1986) reported that LH surge occurred in association with behavioural oestrus in Swamp buffaloes and lasted for 7-12 hr. Peak LH concentrations of 61-126 ng/ml were observed 4-15 hr after the onset of oestrus whereas the interval between LH peak and the end of oestrus was much less variable. Occurrence of an E2 peak before the pre-ovulatory LH surge and a positive correlation between E2 and LH during the 24 hr period before the LH surge (Kanai and Shimizu, 1986) indicate a role of E2 in mediating LH release.

The timing of LH in relation to estrous symptoms and ovulation is more important than the peak itself. Seren et al., 1994 found that the average interval between LH peak to ovulation was 35.5 hrs in buffaloes with a single ovulation and 60 hrs in those with double ovulation. Moioli et al.,(1998) in a study on buffalo estrous behaviour, found a mean interval between peak LH to ovulation of 25.2±13.1 hrs for those animals which became pregnant and 46.1±18, 8 hrs for those which did not become pregnant.

It is now well established that pulsatile secretion of GnRH is essential for the pulsatile release of LH from pituitary gland into circulation. The pulsatile LH secretion is used as an indirect measure of hypothalamic GnRH secretion (Clark IJ., 2002) The pulsatile secretion of LH, and therefore GnRH secretion, has been shown to fluctuate throughout the reproductive cycle depending on steroid status ranging from one pulse per hr during the follicular phase to one pulse every 3-4 hr or longer during the luteal phase (Rahe et al 1980; Walters et al 1984). A reduced LH pulse frequency during the luteal phase as well as reduction in both LH pulse frequency and amplitude during the follicular phase in summer compared to winter in buffalo cows has been observed (Aboul-Ela et al., 1988). Also, the lower LH peak values and circulating FSH levels were observed in summer months (Janakiraman et al., 1980; Razdan et al., 1982).

The LH surge induced by the administration of GnRH in the Ovsynch protocol has a shorter amplitude than the endogenous surge, which may limit the formation and growth of the CL (Chenault et al., 1990; Macmillan et al., 2003). Ambrose et al., (1998), working with non-milking Holstein cows, verified that the prolonged GnRH release promoted by an implants induced an LH surge of greater amplitude stimulating the formation of CLs of greater size and functionality than a single GnRH injection.

In cattle, ovulation takes place 12h after end of estrus and 24h after onset of LH surge (Hafez, 1987). In Murrah buffalo, Paul (2003) observed a large variation of 28–60h

Mechanical stimulation of reproductive tract tends to hasten the LH surge and thus, shortens the interval from estrus to ovulation (Randel et al., 1973). The control of ovulation is brought about by the interactions between the pituitary gonadotropins i.e. FSH and LH and intraovarian factors such as steroids, cytokines and other growth factors (Findley et al.,1996).

2.4 Oestradiol

Estrogens are hormones produced by the ovary (follicle) and transported by carrier proteins, the most important of which is estradiol 17ß (E2). Oestrogens, including estradiol , estrone and estriol, have been called the "female sex hormone". They have the widest range of biological functions, of all steroids. It acts on central nervous system to induce the behavioral estrus, cyclic changes in the female reproductive tract, duct development in the mammary gland, development of secondary sex characteristics in females, potentiating effect on uterine contraction by increasing amplitude and frequency, negative and positive feedback to control of FSH & LH, Calcium uptake and bone ossification and protein anabolic effect (Hafez, 2000; Bearden et al., 2004).

Peripheral plasma E2 profile in buffalo is not very different from that reported in cattle, with peak concentrations observed before and during the preovulatory surge of gonadotropins after which the levels come down to base values in the next few days, with minor fluctuations throughout the estrous cycle. In buffalo the general pattern of secretion of E2 indicates a peak that takes place the day before the LH peak or frequently very close to this with blood levels between 9 and 13 pg/ml in Murrah and Mediterranean buffaloes, respectively. The basal levels of E2 during the luteal phase of the cycle, are between 3 and 8 pg/ml, but lower values can be observed in the early luteal phase (1.0-1.5 pg / ml) (Singh B., 2001; Malfatti, 2003).

During the luteal phase, a minor peak of 10 pg/ml and a more sustained peak of 20 pg/ml were observed on days 4-5 and day 10 after oestrus. These minor peaks might have resulted from waves of follicular growth since buffaloes have been reported to undergo two or three waves of follicular growth during the oestrous cycle, with the second wave occurring during days 10-11 of the cycle (Baruselli et al., 1997; Manik et al., 1998)

Positive feedback effect of E_2 on LH secretion have been observed (Land et al., 1976; Mori et al., 1987) initiating ovulation. The proestrus rise of E_2 may be associated with triggering of LH release by positive feedback on hypothalamo-hypophyseal axis (Batra and Pandey, 1982). Whole milk E_2 concentrations have been reported to be higher and positively correlated with those in plasma during oestrous cycle in buffaloes (Batra et al., 1980; Batra and Pandey, 1982).

Glencross and Pope (1981), working with taurine cattle, found that E_2 levels are low in peripheral plasma for most of the estrous cycle and rise as the concentration of progesterone begins to fall, reaching a peak 3 to 4 days later. Probably the drop in progesterone concentration following luteal regression allows the preovulatory follicle to increase its secretion of E_2 (Karsh et al, 1978). In the Holstein heifers used by Glencross and Pope (1981), plasma E_2 concentration was low at the start of luteal regression (2.2 ±0.5 pg/ml), increased to 3.8 ±0.6 pg/ml the next day and reached 6.6 ±0.9 pg/ml when the concentration of progesterone in the blood had fallen to a minimum. The highest concentration of E_2 (10.1 pg/ml) was recorded one or 2 days after complete luteolysis (Wettemann et al., 1972).

2.5 Progesterone

Progesterone (P_4) is secreted by luteal cells of corpus luteum, also by placenta and adrenal gland. It is transported by a binding globulin as for androgens and estrogen. LH primarily stimulates P_4 Secretion. P_4 prepares the uterus for implantation and maintenance of pregnancy by increasing activity of secretory glands in endometrium and by inhibiting motility of myometrium, acts synergistically with estrogens to induce behavioural estrus, develops alveoli of mammary glands, inhibits estrus and ovulatory surge of LH at higher levels (Hafez, 2000). Several studies have been reported on peripheral P4 concentrations during estrous cycle in buffalo (Shah and Mehta, 1992; Kamboj and Prakash, 1993; Mondal et al.,2001; Mondal and Prakash, 2002; Mondal et al., 2003) showing that peripheral plasma P4 profile in buffalo is very similar to that in cattle. P4 levels rise and fall in coincidence with the growth and regression of corpus luteum (CL) since CL is the source of P4 in cycling buffalo (Ahmed et al., 1977).

Peripheral P4 concentrations are lower on the day of oestrus (0.1 ng/ml), rise to peak concentrations of1.6-3.6 ng/ml on days 13 to 15 of the cycle (Ahmed et al., 1977; Bachalaus et al., 1979; Seren et al., 1994) or even on day 17 (Pahwa and Pandey, 1983) before declining to basal levels at the onset of next estrus. It goes down 2 or 3 days before the peak of LH, begin to rise 2 to 4 days after the LH peak while the highest values are characteristic of the luteal phase (5-12 ng/ml and 4-6 ng/ml in Mediterranean and Murrah buffaloes respectively). P4 levels continue to increase in animals that conceive but drop 3 days before the next oestrus in those that fail to conceive (Batra et al., 1979). The onset of the decline in P4 concentrations is variable, depending upon the time of regression of CL.

Peripheral P4 concentrations may change during the seasons (Srivastava et al., 1999) i.e. lower P4 levels at oestrus as well at midluteal phase in hotter (0.14 ± 0.05 and 2.05 ± 1.16 ng/ml, respectively) than in cooler months (0.49 ± 0.06 and 3.11 ± 0.20 ng/ml, respectively) are believed to be responsible for the poor expression of oestrus and low conception rate during summer season by some authors (Rao and Pandey, 1982). Contrary to this, others have, observed P4 concentrations to be significantly higher during summer compared to those in winter season (Mondal et al., 2001; Mondal et al., 2004). The pattern of P4 concentrations in milk has been found to be similar to that in plasma, although the concentrations in milk are much higher than those in plasma (Kamboj and Prakash, 1993). The average concentration of P4 in milk, which was 0.5 ng/ml at oestrus increased to 18 ng/ml on day 15 and declined thereafter to 4.4 ng/ml 3 days preceding the next oestrus in non pregnant buffaloes (Batra et al., 1979). P4 concentrations in ovarian follicular fluid are much higher than those in peripheral circulation (Prakash et al., 1997; Palta et al., 1998).

2.6 Physiological Parameters

Perera, (2001) reported respiration rate (RR) (15-20 breaths per min) and pulse rate (PR) (40 - 45 per min) for buffalo. Pulse rate is assessed by palpation of coccygeal artery. Respiratory rate is estimated by visual observation of chest and abdominal movements or observing inhalation and exhalation through nostrils. Brief end-inspiratory pauses, during which the respiratory system remained inflated above the end-expiratory level, have been observed in animals of several species, and quite frequently in cattle and buffalo, besides neonates and some other animals (Mortola, 2001).

RR and PR shows a positive correlation with other physiological responses (rectal temperature and skin temperature) and biochemical parameters (alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and cortisol) and temperature humidity index (THI) (Mclean, 1963; Chandra Bhan et al., 2012). The respiration rate increases when environmental temperature increases (Bond and McDowell, 1972). Highly positive correlation was reported between the respiration rate and ambient temperature and it even raised upto 0.833 when humidity was constant in buffaloes (Misra et al., 1963; Findlay and Ingram, 1961). Radadia et al., (1980) observed a positive correlation (r=0.234- 0.768) between ambient temperature and respiration and pulse rates in buffaloes. Gangwar et al., (1988) reported that environmental temperature has significant relation with the variation in the pulse rate. The result of their studies indicated that average values of pulse rate were higher during summer and lower during winter. Joshi et al., (1982) reported that pulse rate increased moderately during exposure to hot environment in buffaloes. Relationship among environmental parameters and animal physiological parameters had been observed by Pagthinathan et al, (2002) where they found vaginal temperature, rectal temperature and pulse rate were not significantly correlated to relative humidity. However, respiratory rate was negatively correlated to relative humidity. This reflects the negative impact of relative humidity on respiratory evaporation (Bearden and Fuguay, 1997).

The sensitivity of buffalo to neuro-endocrine response as a result of clitoral stimulation may result in general excitement, which could be reflected in physiological parameters like pulse and respiration and hence proposed to be investigated.

Materials & Methods

3 MATERIALS AND METHODS

3.1 Experimental Animals

A total of 92 apparently healthy female Murrah buffaloes maintained at the Institute cattle & buffalo Farm, LPM Section, IVRI, Izatnagar, Uttar Pradesh , were utilized for this investigation during the breeding season from September 2014 to February 2015.The farm is located at an altitude of 166 meters above the mean sea level on 28.35°N latitude and 79.41°E longitude.

3.2 Housing and Management

The animals were maintained under isomanagerial conditions with intensive system and housed in a well ventilated brick cemented house with non-slippery floor and open byre and offered standard ration having green and dry fodder along with concentrate and *ad libitum* clean drinking water.

3.3 Experimental design

Estrus was identified on the basis of teaser bull parading twice daily and visual observation of estrus signs (Jainudeen and Hafez, 2000). Estrus animals were inseminated twice as per AM-PM schedule, using good quality frozen semen. 92 experimental buffaloes were first divided in parous (n=68) and heifer (n=24) categories, to examine the effect of parity. Further, parous animals were subdivided into treatment (n=34) and control (n=34) groups. Similarly heifers were subdivided into treatment (n=14) and control (n=10) groups. Animals in treatment group were given thirty seconds of clitoral stimulation (massage) after artificial insemination while no such stimulation was given in control animals. Buffaloes that failed to settle in first AI were not used subsequently.

Experimental groups	Number of Animals		
	Total	Parous	Heifer
Control	44	34	10
Treatment	48	34	14
Total	92	68	24

3.4 Clitoral stimulation

Clitoral stimulation in treatment group was carried out by placing the forefinger and thumb on the embedded portion of clitoris located at the ventral commissure of vulva and then applying gentle massage for 30 seconds.

3.5 Physiological response

Physiological response in experimental animals in terms of pulse and respiration rates were recorded Pre-AI (10-15 minutes before A.I) and Post A.I (within 5 minutes of A.I.) in both the groups viz. control (n=25) and treatment (n=27). Pulse rate was assessed by palpation of middle coccygeal artery and respiratory rate was estimated by visual observation of chest and abdominal movements and observing inhalation and exhalation through the nostrils.

3.6 Blood Sampling

For serum LH and estradiol assay, three buffaloes were taken from each group. Eight samples were collected from each animal on the day of estrus. First blood sample was collected 1 hr before AI followed by rest of the samples at hourly interval. Blood (5 ml) was collected by juglar venipuncture in sterile tubes. Serum was separated by centrifugation at 3000 rpm for 15 min and stored at -20^{o}C until analysis.

For serum progesterone assay, blood samples were collected on day of estrus (day 0), 6th ,12th and 18th day post AI (n=6/ group).

Location of clitoris

Clitoral stimulation

3.7 Serum Hormonal assay

3.7.1 Luteinizing Hormone

Serum LH concentration was estimated using Bovine LH ELISA test kit (Endocrine technologies, inc., USA)

Principle

The LH ELISA test kit is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a polyclonal anti-LH antibody for solid phase (microtiter wells) immobilization and a mouse monoclonal anti-LH antibody in the antibody enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in LH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 2 hour incubation at 37^0C, the wells are washed with water to remove the unbound-labelled antibodies. A solution of TMB (3,3',5,5'-Tetramethylbenzidine) is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of 2N HCL, and the absorbency is measured spectrophotometrically at 450 nm. The intensity of the color developed is proportional to the amount of enzyme present and is directly related to the amount of unlabelled LH in the sample. By reference to a series of LH standards assayed in the same way, the concentration of LH in the unknown sample is quantified.

Procedure

1. Desired numbers of coated wells in the holder were secured.
2. 50µl of standards, specimens and controls were dispensed into appropriate wells.
3. 100µl of enzyme conjugate was dispensed into each well and mixture was shook well for 30 seconds following incubation at 37^0C for 2 hours.
4. The incubation mixture was removed by flicking the plate contents into a waste container.
5. The microtiter wells were rinsed and flicked 5 times with wash buffer.

6. All residual water droplets were removed by striking the wells sharply onto absorbent paper or paper towels.
7. 100µl of solution of TMB was dispensed into each well and gently mixed for 10 seconds, followed by incubation at room temperature for 20 minutes, in the dark.
9. The reaction was stopped by adding 50µl (one drop) of 2N HCL to each well.
10. The solution was gently mixed for 30 seconds. It is important to observe a color change from blue to yellow.
11. Optical Density was read at 450nm with a microtiter well reader.

Calculation of result and sensitivity

The mean absorbency values (A450) for each set of reference standards, specimens, controls and patients sample were calculated. A standard curve was constructed by plotting the mean absorbency obtained from each reference standard against its concentration in ng/ml on graph paper, with absorbency values on the vertical or Y axis, and concentrations of LH on X axis. Using the mean absorbency values for each specimen, the corresponding concentration of BOVINE LH from the standard curve was determined.

The minimal detectable conc. of BOVINE LH by this assay was estimated to be 0.1 ng/ml.

3.7.2 Estrogen and Progesterone:

Radioimmunoassay (RIA) has been the most important advancement in endocrinology in the past 50 years. It is possible to measure low concentration of hormone in large number of samples rapidly through this assay. Recent advancement in reproductive endocrinology and neuroendocrinology are principally attributed to RIA which is a standard and highly sensitive method to assay almost all reproductive hormones. Progesterone and estrogen in serum samples was estimated by RIA technique using diagnostic I^{125} kits supplied by Immunotech, France.

Principle:

The principle of RIA is based on the fact that in the absence of unlabelled antigen or hormone, the labeled radioactive hormone has maximal opportunity to react with limited number of antibody binding sites. Antibodies have bivalent binding sites so one antibody molecule may bind two hormone molecules. If some of the limited antibody binding sites are allowed to react with the unlabelled hormone lesser site will be available for labeled hormone and this results in decreased antibody bound radioactivity. Amount of hormone in the unknown samples is determined by comparing with standard curve.

Procedure:

The desired sera and reagents were allowed to reach to room temperature. A volume of 100 µl (for estradiol)/ 50 µl (for progesterone) of standard and samples were taken into antibody coated tubes and 500 µl of tracer was added to each tube thereafter. Separately a volume of 500 µl of tracer was added to two additional uncoated plane tubes in order to get the total counts. The tubes were vortexed gently, covered with paraffin and incubated for 3 hours for estrogen and 1 h for progesterone, respectively at 18-25^{0}C. The contents of all the tubes were decanted out except the tube prepared for total count. The radioactivity of coated tubes were then counted for 1 min. / per tube in auto gamma counter (Cobra-II, R-Packard Bioscience Company, Germany) calibrated for I^{125} and programmed to estimate the concentration of samples from the standard curve.

Analytical sensitivity of kit for estimation of progesterone was 0.3 ng/ml; for estradiol <6 pg/ml, respectively. The intra and inter-assay coefficient of variation of progesterone was <8 and <12.0%, for estradiol 12.1 and 11.2%, respectively.

3.8 Conception Rate

Pregnancy diagnosis was carried out at day 75-90 of breeding in non return buffaloes and Conception Rate was calculated as per the following formula :

$$\%\text{Conception Rate (CR)} = \frac{\text{No. of animals Pregnant}}{\text{Total no. of Inseminations}} \times 100$$

3.9 Service per conception

Service per conception was calculated as per formula:

$$= \frac{\text{Total no. of Inseminations}}{\text{No. of animals Pregnant}} \text{X } 100$$

3.10 Statistical Analysis

Paired T-test was applied for pulse and respiration rate. Repeat measure ANOVA was used for hormones. Chi square test was used for finding the association between clitoral stimulation and CR in the buffalo. Odds of pregnancy following clitoral massage in relation to control were also calculated. SAS version 9.3 was used for data analysis. Analysis for LH was done using Graph Pad Prism 4.00 software.

Results & Discussion

4 RESULTS AND DISCUSSION

The present investigation was conducted to examine the effect of clitoral stimulation after insemination on physiological parameters, hormonal profile and conception rate in buffaloes.

4.1 Physiological parameters

4.1.1 Pulse rate

The mean pre and post AI pulse rate recorded in control buffaloes were 61.44±1.29 /min and 61.84±1.23 /min respectively. The mean pre and post AI pulse rate recorded in treatment buffaloes were 62.81±1.22 /min and 65.63±1.13 /min respectively. (Table 1)

There was no significant difference in pulse rate in the control buffaloes Pre and Post AI. Similarly values didn`t differ between control and treatment groups before A.I.

Within treatment group, a significant ($p<0.05$) increase in pulse rate was recorded Post A.I as compared to pre-AI, which was also evident as compared to post A.I. values of control group.

Table 1: Effect of clitoral stimulation on pulse rate (per minute) of experimental buffaloes (Mean ± SEM)

Groups	Pre AI	Post A.I
Control	61.44±1.29	61.84^{a}±1.23
Treatment	62.81^{A}±1.22	65.63^{bB}±1.13

Means with different superscript (a,b) in column differs significantly ($P<0.05$)
Means with different superscript (A,B) in a row differs significantly ($P<0.05$)

4.1.2 Respiration Rate

The mean pre and post AI respiration rate recorded in control buffaloes were 10.00±0.45/min and 10.24±0.42 /min respectively. The mean pre and post AI respiration rate recorded in treatment buffaloes pre-A.I were 10.44±0.45/min and 12.29±0.44/min respectively. (Table 2)

There was no significant difference in respiration rate in the control buffaloes Pre and Post AI. Similarly values didn`t differ between control and treatment groups before A.I.

Within treatment group, a significant ($p<0.05$) increase in respiration rate was recorded Post A.I as compared to pre-AI, which was also evident as compared to post A.I. values of control group.

Table 2: Effect of clitoral stimulation on respiration rate (per minute) of experimental buffaloes (Mean ± SE)

Groups	Pre AI	Post A.I
Control	10.00±0.45	10.24^{a}±0.42
Treatment	10.44^{A}±0.45	12.29^{bB}±0.44

Means with different superscript (a,b) in column differs significantly ($P<0.05$)
Means with different superscript (A,B) in a row differs significantly ($P<0.05$)

Comparison of pre and post AI PR ($P=0.029$) and RR ($P=0.0013$) revealed that there was a significant increase in the treatment group. However, in the control group, neither the PR nor the RR was significantly affected indicating that AI did not affect these vital parameters *per se.* The values were comparable in the control and treatment buffaloes before AI., however they were differing significantly($P<0.05$) Post-AI.

The significant elevation in PR and RR in the treatment group suggests that the clitoral massage causes sympathetic activation.

Clitoris is innervated by pudendal nerve and well-supplied with sensory nerve endings or afferent fibres, therefore stimulation of clitoris might be responsible for sympathetic activation and increase in PR and RR.

Since no such study is available in literature hence findings could not be compared upon with other works. However, further detailed studies are warranted with regard to understanding the sensitivity of buffalo to neuro-endocrine response as a result of clitoral stimulation resulting in general excitement.

4. 2 Luteinizing Hormone

Mean serum LH concentration (ng/ml) on day of estrus was ranging between 0.43 – 6.58 and 0.36 – 15.83 in control and treatment groups respectively. Peak serum LH concentration in control was 6.58±5.64 ng/ml at 6th hr or 5 hrs after A.I. and that of treatment group was 15.83±14.578 ng/ml just after the clitoral stimulation after A.I. i.e. at 1 hrs. Mean LH concentration in control and treatment group was 2.327± 0.84 and 6.405 ± 2.64 ng/ml respectively. (Table 3 and Fig. 1)

There was no significant effect of time on the LH level either in the control or treatment group. Similarly, the interaction between the clitoral stimulation treatment and time was not significant. Overall, there was a significant ($P<0.05$; $F=9.948$) increase in the mean LH level in treatment group (6.405±2.64 ng/ml) as compared to control (2.37± 0.838 ng/ml) irrespective of the sampling time. This is due to the fact at every time point tested except 4th & 6th hr, treatment group had higher values of LH than that of control.

Table 3: Effect of clitoral stimulation on Serum LH (ng/ml) in experimental buffaloes (Mean ± SE)

Time (hrs)	Control	Treatment	P Value
0	2.74±1.25	0.67±0.08	0.8073
1	1.36±0.30	15.83±14.57	0.0934
2	0.87± 0.25	3.91±3.43	0.7194
3	0.48± 0.097	11.79± 11.28	0.1858
4	0.43±0.19	0.39±0.035	0.9963
5	4.41±3.61	6.51±6.06	0.8035
6	6.58±5.64	0.367±0.046	0.4629
7	1.72±1.23	11.74±11.13	0.2404
Mean	2.327±0.838	6.405±2.64	0.0344

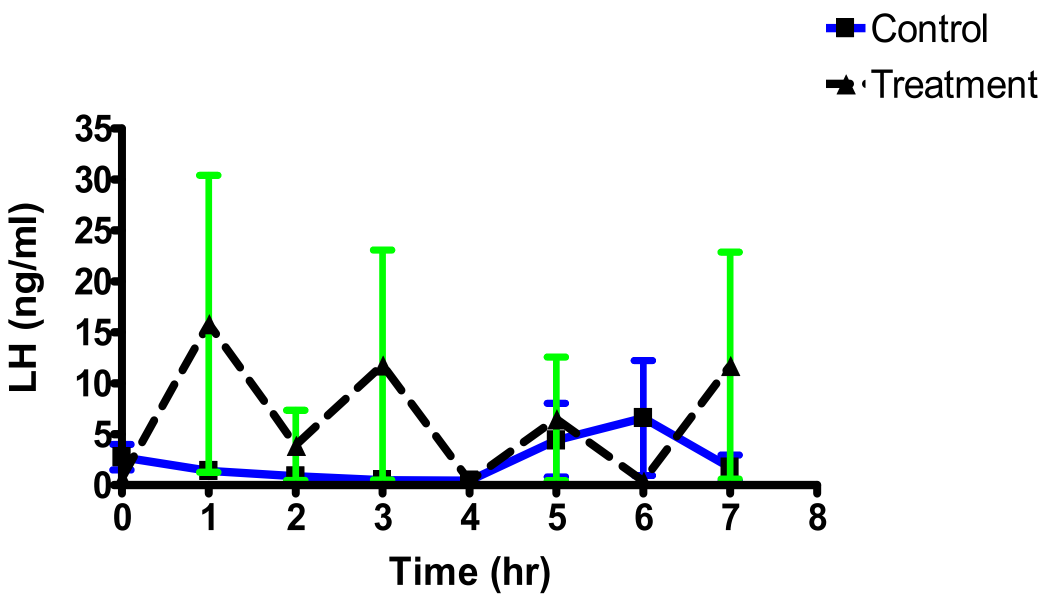

Fig.1: Effect of clitoral stimulation on Serum LH concentration (ng/ml) in experimental buffaloes

Individual Animal graphs (fig 2-4) shows that the amplitude and frequency of LH was higher in treatment animals as compared to control. The LH conc. rose up to highest 45 ng/ml within 1hr after clitoral massage in one of the treated animals. Peak LH conc. of animals in the treatment group was higher and the peaks occurred within 5 hours of stimulation presumably resulting in the better synchrony among insemination-ovulation-fertilization. The LH concentrations were at basal levels till 5^{th} hr in control animals and peaks were seen after 5 hours of AI.

The mean peak LH levels in control buffaloes obtained in our study (6.5± 5.64 ng/ml) are in resemblance to the reports of Kaker *et al.* (1980), Avenell *et al*. (1985) and Kanai and Shimizu, (1984) but are lower than those reported by Madan & Johnson (1973), Kaltenbach *et al.* (1974), Lemon *et al*. (1975), Heranjal *et al* (1976), Batra and Pandey (1982) and Kanai and Shimizu (1984).

The data available on the concentrations of gonadotrophic hormones in blood of buffalo indicate that the temporal patterns of LH and FSH are basically similar to those in cattle (Avenell *et al*., 1985; Kanai *et al*., 1990). The LH concentrations in control buffaloes at the time of oestrus period are consistent with observations on cattle (Henricks *et al*., 1970; Madan & Johnson, 1973; Schams *et al.,* 1977; Dobson, 1978). However, large variations in the peak LH levels of cattle have been reported by different workers. In our study, the mean basal LH concentration was 0.43±0.19 ng/ml in control, which is comparable to the basal values reported for cattle.

Clitoral cauterization applied under epidural anaesthesia in healthy dairy cows did not affect the mean LH concentration, LH peak concentration and mean duration of LH surge (Yüksel and Deveci, 2011) indicating that clitoris is not inevitable for LH release and concentration however present study demonstrates that its stimulation leads to augment response.

Peak value of LH in buffaloes at estrus ranges between 20-35 ng/ml (Batra and Pandey, 1982; Kanai and Shimizu, 1984). Heranjal *et al.* (1976) reported peak

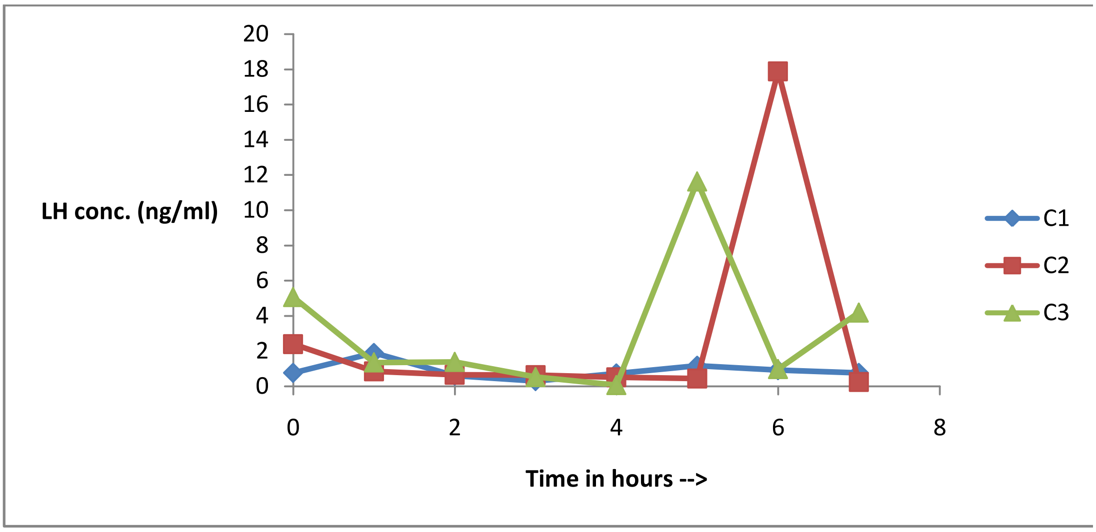

Fig 2 LH conc. in control animals

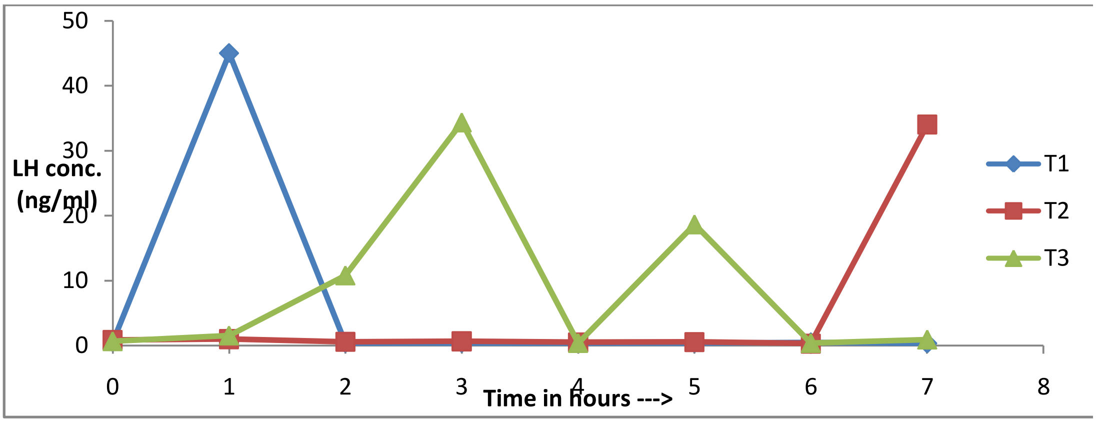

Fig: 3 LH concentrations in treatment animals

Fig. 2-4 Effect of clitoral stimulation on Serum LH conc. in experimental buffaloes (Individual animal values)

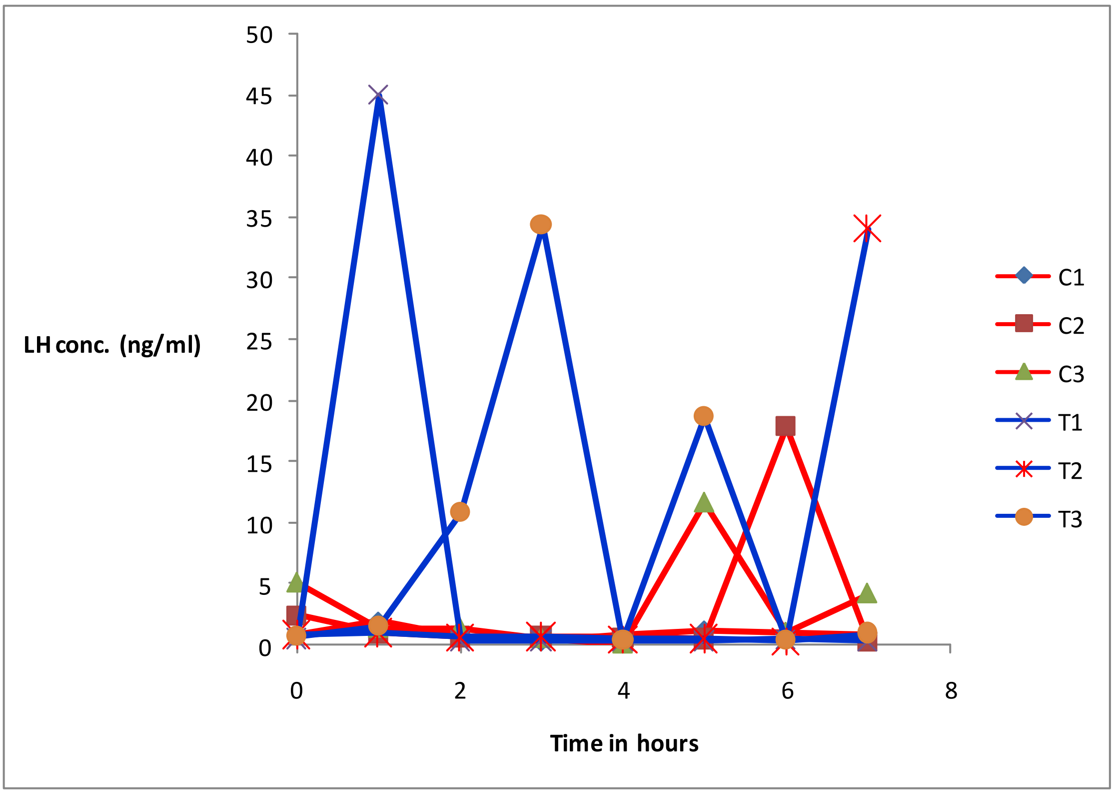

Fig: 4 Effect of clitoral stimulation on LH conc. in control (c) and treatment (t) animals

LH levels between 14.03-38.5 ng/ml at estrus in 7 Murrah buffaloes. In our study peak LH concentrations ranged from 18.64 to 45 ng/ml, which is considerably higher than that of Heranjal *et al.* (1976) and other researchers, indicating the positive effect of treatment in same breed of animals.

The peak LH values after clitoral stimulation in our study are higher than that reported in cattle. Mechanical stimulation of reproductive tract tends to hasten the LH surge and thus, shortens the interval from estrus to ovulation (Randel et al., 1973).

Actual LH surge could not be predicted due to difficulty of detecting exact time of onset of heat in the animals because only twice a day teaser bull parading was done. However, potentiation on LH secretion upon clitoral stimulation was exhibited in present study.

4.3 Serum Estradiol

Serum E_2 Concentration was determined in both the groups on day of estrus at hourly intervals to examine the effect of clitoral stimulation. Mean serum E_2 concentration on day of estrus was ranging between 8.09 – 16.04 and 6.35 – 21.26 pg/ml in control and treatment groups respectively. Mean E_2 conc. in control and treatment group was 12.693±1.28 and 13.882±1.75 pg/ml respectively. (Table 4)

Table 4: Effect of clitoral stimulation on Serum E_2 (pg/ml) in experimental buffaloes (Mean ± SE)

Time hrs	Control	Treatment	P Value
0	14.07±6.40	12.83±6.46	0.8402
1	12.69±4.56	20.97±5.95	0.1844
2	14.04±4.87	18.19±4.26	0.5016
3	15.50±1.06	11.60±2.60	0.5276
4	16.08±4.84	21.26±8.07	0.4025
5	8.09±3.38	11.19±0.55	0.6149
6	10.17±0.79	8.64±2.11	0.8036
7	10.89±1.89	6.35±0.40	0.4631
Mean	12.693±1.28	13.882±1.75	0.5857

There was no significant difference between E_2 concentrations of control and treatment groups on different sampling times, indicating neither clitoral stimulation nor sampling time significantly affected the levels of estradiol in the experimental buffalo.

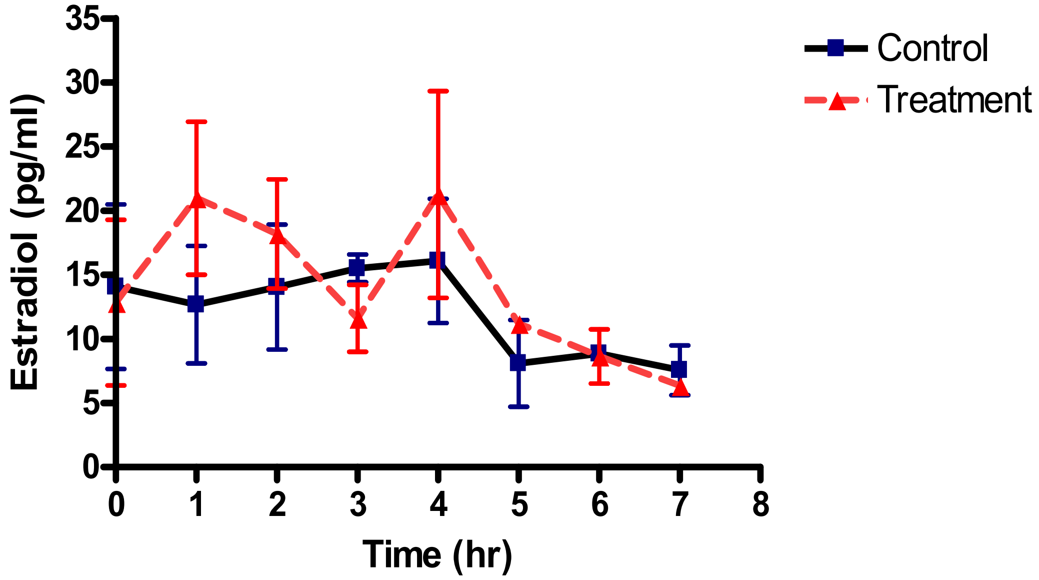

Fig. 5: Effect of clitoral stimulation on Serum estradiol concentration (pg/ml) in experimental buffaloes (Mean±SE)

The pattern of serum E_2 and occurrence of its peak around estrus (16.08 ± 4.84 pg/ml) in control group in the present study is in accordance with the earlier reports (Singh *et al*., 2001; Malfatti, 2003). Serum E_2 concentration in control group in our study on the day of estrus are in resemblance with Bachalaus *et al.* (1979), Kanai and Shimizu (1984), Avenell *et al*. (1985) and Arunji (2008), but are lower than those reported by Batra and Pandey (1982, 1983). However, peak concentrations of estradiol 30–35 pg/ml were detected on the day of estrus or a day before (Batra and Pandey, 1982), followed by a decline to 5–10 pg/ml within two days which is indicative of enhanced estradiol production by the preovulatory follicle during proestrus. The serum concentration of oestradiol-17β in buffalo during the follicular phase of the estrous cycle appears to be relatively less than that in cattle (Avenell *et al*., 1985; Kanai *et al*., 1990; Roy and Prakash, 2009). Although this has been suggested as a possible reason for the lesser intensity of estrus exhibited by buffaloes.

Our results are in accordance with Bozkurt *et al,* (2007) who observed non significant increase in E_2 concentration after clitoral massage in dairy cattle. However, there is no evidence as to whether clitoral stimulation affects steroid hormones in buffaloes.

4.4 Serum Progesterone

Serum P_4 concentration in control and treatment group at 0, 6,12 and 18 days ranged between 0.69±0.10 - 4.04±0.61 and 0.823±0.10 - 6.75±1.04 ng/ml respectively. Lowest values were recorded on the day of estrus in both the groups. No significant difference was observed between both the groups on different sampling days, except at day 18 when significantly $P<0.05$) higher levels of P_4 were detected in treatment group as compared to control (6.75±1.04 Vs 4.04±0.61 ng/ml). (Table 5 and Fig. 6)

A significant group effect on P_4 level was seen on day 18. This could be due to more number of buffaloes became pregnant in the treatment group (5/6) as compared to control (3/6) as P_4 levels continue to increase in pregnant while starts declining in non-pregnant animals, due to the activation of endogenous luteolytic mechanism.

Progesterone showed a significant time effect on day 12 and 18 post-breeding which is due to the effect of luteal phase. The interaction between clitoral stimulation treatment and time was not significant.

Table 5: Effect of clitoral stimulation on Serum progesterone (ng/ml) in experimental buffaloes (Mean ± SE)

Day	Control	Treatment	P Value
0	0.690±0.10	0.823±0.10	0.8567
6	1.84±0.49	1.94±4.34	0.8980
12	3.15±0.53	3.38±0.29	0.7630
18	4.04±0.61	6.75±1.04	0.0006

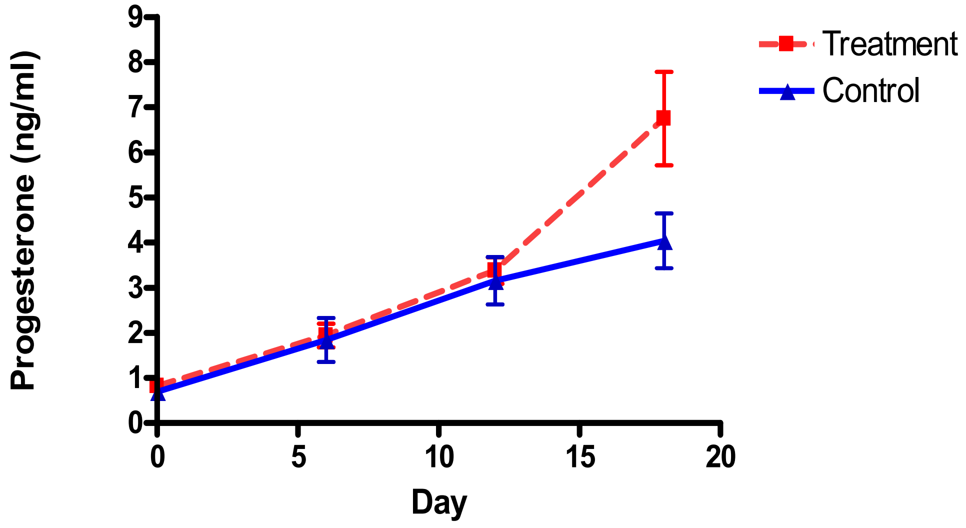

Fig. 6: Effect of clitoral stimulation on Serum P_4 concentration (ng/ml) in experimental buffaloes

The results obtained in control group are in accordance with other workers (Ahmed et al., 1977; Bachalaus et al., 1979; Batra et al., 1979: Seren et al., 1994; Pahwa and Pandey, 1983) that P_4 Varies from 0-6 ng/ml during the cycle, being at basal levels during estrus and rises thereafter. The changes in concentrations of progesterone in blood and milk during estrous cycle are similar to those in cattle, but the peak concentration is relatively less (Dobson and Kamonpatana, 1986; Perera *et al.,* 1987; Singh *et al*., 2001)

4.5 Conception Rate

In present study, the effect of clitoral stimulation after AI was examined on conception rate in buffaloes. Experimental buffaloes were further divided in parous and heifer categories, to examine the effect of parity. The CR in parous buffaloes in treatment group (67.65%) was found to be significantly (P<0.01) higher as compared to control (32.35%). The CR in treated heifers (66.67%) was also higher than control (41.67%) but statistical significance could not be obtained. However, the pooled conception rate in the treatment group (67.39%) was found to be significantly (P<0.01) higher as compared to control (34.78%). (Table 6-8 and Fig 7).

Table 6: Effect of clitoral stimulation on conception rate in experimental buffaloes (parous)

GROUPS	CR (%)	Chi-Square & P value	Odds Ratio with95% CI
TREATMENT	67.65 (23/34)*	DF1 Value 8.471 P = 0.0035	4.372 (1.582 to 12.08)
CONTROL	32.35 (11/34)*		

*Figures in the parenthesis indicate no. of buffaloes pregnant out of total inseminations

Table 7: Effect of clitoral stimulation on conception rate in experimental heifers

GROUPS	CR(%)	Chi-Square & P value	Odds Ratio with95% CI
TREATMENT	66.67 (8/12)*	**DF**1 **Value 1.510** P = 0.2191	**2.8000** (0.5318 to 14.74)
CONTROL	41.67 (5/12)*		

Table 8: Effect of clitoral stimulation on conception rate in Buffaloes (pooled)

GROUPS	CR (%)	Chi-Square & P value	Odds Ratio with95% CI
TREATMENT	67.39 (31/46)*	DF1 Value 9.787 P= 0.0018	3.875 (1.631 to 9.205)
CONTROL	34.78 (16/46)*		

*Figures in the parenthesis indicate no. of buffaloes pregnant out of total inseminations

Service per conception:

In our study, just 1.48 inseminations were required for a conception in parous treatment group while it was 3.09 in control. In heifers, 1.5 inseminations were made to make an animal conceive in treatment group as compared to 2.4 inseminations in control. The overall service per conception in treatment and control buffaloes was 1.48 and 2.88, respectively (Table 9). Low no. of service per conception in treatment group indicates that this will have a beneficial effect on reducing calving interval and finally the lifetime production and reproduction of buffalo, besides reducing the cost of AI.

Table 9: Effect of clitoral stimulation on Service per conception in experimental buffaloes

Groups	Total inseminations	Pregnant	Service/ conception
Parous			
Treatment	34	23	1.48
Control	34	11	3.09
Heifer			
Treatment	12	8	1.5
Control	12	5	2.4
Pooled			
Treatment	46	31	1.48
Control	46	16	2.88

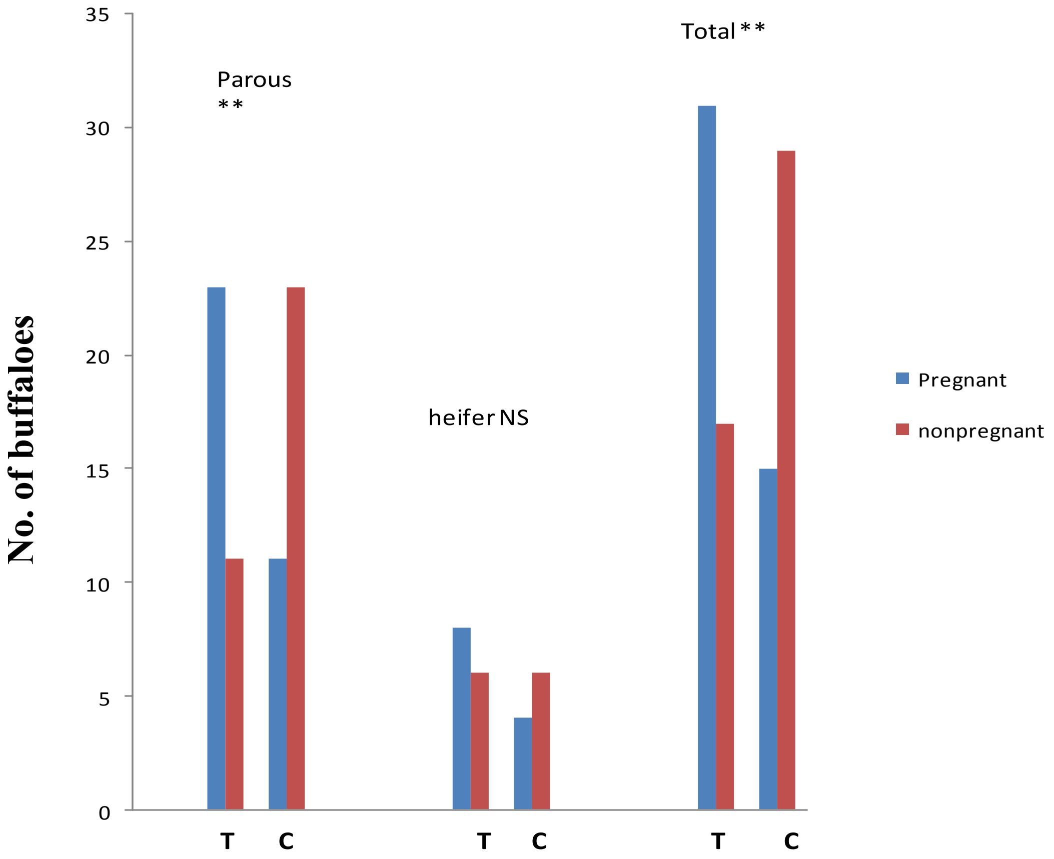

Fig. 7: Number of animals pregnant and non-pregnant in parous, heifer and pooled categories in experimental buffaloes.

It is evident that the effect of clitoral stimulation on the conception rate was highly significant in the parous buffalo. The CR was non significantly higher in the treated heifers. The overall CR was also highly significant in the group of buffaloes that received clitoral massage at the time of AI. The results are supported by the fact that the overall odds of conception in a buffalo that received clitoral massage at the time of AI was 3.875 times high as that of unstimulated control. The odds of getting pregnant after clitoral stimulation in parous buffalo was 4.37 (1.582 to 12.08; 95% C.I.) as compared to parous buffaloes without clitoral stimulation and The odds of getting pregnant after clitoral stimulation in heifers was 2.80 (0.531 to 14.74; 95% C.I.) as compared to heifers without clitoral stimulation.

It is tempting to speculate that the significant increase recorded in the LH concentration in treatment group could be one of the reasons behind the significant increase in the CR in the clitoral stimulated buffalo.

Results on the conception rate is in accordance with earlier work in the cattle (Randel et al., 1975) which demonstrated that clitoral stimulation should be used as a routine procedure following AI in beef cattle. In Philippine swamp buffalo (Carabaos), Capitan et al (1992) found a significant increase in conception rate both in heifers and parous buffaloes after clitoral massage of 30 seconds. Cooper and Foote (1986), Rodriguez (1994) and Arbeiter et al. (1985) reported that clitoral stimulation significantly increased pregnancy rates in cattle heifers as compared to without stimulation. The failure to obtain a statistical significance in positive response to clitoral massage on CR in heifers under present investigation might be due to the lower number of observations. Glauber (1989) also found that conception rates were higher in cows on which clitoral massage was carried out for 10 seconds (65.08%) than in controls (51.43%). Lunstra et al. (1983) reported that clitoral stimulation increased the first service pregnancy rate in cows.

Clitoral stimulation at the time of A.I have been reported as an effective means of increasing pregnancy rate in dairy cows (Singh et al., 2001; Lunstra et al., 1983). However, Rodriguez et al. (1980) showed that pregnancy rate was higher in animals not receiving clitoral massage than in those receiving it. Many experimental or practical conditions may differ from the conditions that existed when these data were collected and alter response to clitoral stimulation.

Clitoral massage shortens the estrus duration in crossbred cattle herds with prolonged oestrus, in terms of number of days of expression of estrus signs. This in turn reduces the necessity for double AI and improves the conception rate of single AI, lowering number of services preconception (Kutty, 2006). Arbeiter et al. (1985) observed a more rapid follicle growth and ovulation earlier in clitoral massage group compared with the control group. These data in cattle and swamp buffalo and our study in riverine buffalo demonstrate that clitoral massage following artificial insemination increases conception rate, in bovines. The information generated in present investigation fulfils the critical gap that existed in riverine buffalo.

From our study, we may not exactly elucidate the mechanism behind the increase in CR in buffaloes receiving clitoral stimulation following artificial insemination, except the finding positive effect of stimulation on endogenous LH release. Various neural pathways exists between the reproductive system and hypothalamic-pituitary axis (Frandson, 1975;Hafez et al., 2000). Stimulation of the reproductive tract at artificial insemination hastened the LH surge and ovulation in the cow (Randel et al., 1973). Sperm transport may be the point of greatest action as stimulation of the reproductive tract has been shown to affect uterine motility (VanDemark and Hays, 1952 Hays and VanDemark, 1953a, b). Clitoral stimulation at the time of A.I. causes oxytocin release from the posterior pituitary gland, which has positive effect on the sperm transported into female genital tract (Bozkurt *et al*., 2007). Oxytocin was used to increase CR by improving the sperm transport in the female reproductive tract of several species (Sayre and Lewis 1997; King et al., 2004; Yildiz, 2005). This indicate a neural involvement with pituitary release of oxytocin from stimulation of the reproductive tract at the time of breeding.

It appears from the facts that clitoral stimulation affects pituitary release of oxytocin and L.H in the bovines and thereby ovulation time. These factors may directly or indirectly affect conception rate. Moreover clitoral massage is an inexpensive managerial practice which may simulate the natural service as in general conception from natural service is more than that of A.I.(Jainudeen and Hafez 1993; Agarwal and Shanker 1994). The result of present study clearly indicates that clitoral stimulation at the time of AI in buffaloes has a significant positive effect on endogenous LH release, presumably influencing the ovulation time and thereby augmenting CR. Hence it can be recommended that clitoral stimulation at the time of AI may be adopted as a package of practice in bovines, more particularly in buffaloes as the national figure of CR from AI in field condition needs to be enhanced to exploit the fullest potential of this versatile species.

Summary & Conclusion

5 Summary and Conclusions

The riverine buffalo (*Bubalus bubalis*) is an important livestock resource for meeting food requirements to human population. This species contributes 86.57 million tons of milk out of which India's contribution is the highest (59.21%) as well as the South-Asian region supports 93.38% of the world buffalo population of 177.25 million heads. Preference of Indian farmers is now changing from cattle to buffalo at very faster rate which is evident from 7.99% growth rate in female buffalo population and more than 51 per cent of the milk now flows from the udders of buffaloes although buffalo population is less than half (42.4%) of total cattle population (19^{th} Livestock census, 2012). In the last two decades, buffaloes farming has been popularized and widely expanded in Mediterranean area, Latin America and European countries. Buffalo has a high productive potential often blamed for poor reproductive performance reflected by various inherent problems like delayed sexual maturity, silent estrus, reproductive seasonality, postpartum anoestrus and longer inter-calving intervals. One of the major cause for reduced reproduction potential upon AI is ovarian dysfunction, hormonal imbalance and delayed ovulation. Buffalo has a higher incidence of delayed ovulation than cattle and the conception rate to AI is generally lower. Under field condition CR through Chilled and Frozen semen has been reported very low (25-37.3%).

The study was planned to explore managemental aspect using non hormonal approach that may be applied in buffalo species to overcome this hurdle and C.R may be increased, simply by manual stimulation of clitoris after A.I, with the objectives to investigate the effect of clitoral stimulation after A.I. on physiological parameters, reproductive hormone profiles (luteinizing hormone, estradiol and progesterone) along with conception rate in buffaloes. The study was carried out at the Cattle and Buffalo Farm of the Institute during the breeding season from rate in buffaloes. The study was carried out at the Cattle and Buffalo Farm of the Institute during the breeding season from September 2014 to February 2015. It was conducted on 92 apparently healthy female Murrah buffaloes maintained under isomanagerial conditions with intensive system and housed in a well-ventilated brick cemented house with non-slippery floor. Estrus was identified on the basis of teaser bull parading twice daily and visual observation of estrus signs. Estrus animals were inseminated twice as per AM-PM schedule, using good quality frozen semen. 92 experimental buffaloes were first divided in parous (n=68) and heifer (n=24) categories, to examine the effect of parity. Further, parous animals were subdivided into treatment (n=34) and control (n=34) groups. Similarly heifers were subdivided into treatment (n=12) and control (n=12) groups. Animals in treatment group were given thirty seconds of clitoral stimulation (massage) after artificial insemination while no such stimulation was given in control animals. Buffaloes that failed to settle in first AI were not used subsequently.

Physiological response in experimental animals in terms of pulse and respiration rates were recorded before and after clitoral massage in treatment group (n=27) and before and after AI in control group (n=25).

Hourly blood sampling was done for LH and estradiol assay in three buffaloes from each group. Eight samples were collected. First blood sample was collected 1 hr before AI and other samples at hourly interval. Blood was collected by juglar venipuncture followed by serum separation and storage at -20^{o}C till use. Progesterone was estimated on day of estrus(day 0), 6th , 12th and 18th day post AI (n=6/ group). Serum LH concentration were determined using Bovine LH ELISA test kit (Endocrine technologies, inc., USA) while ovarian hormone (E_2 and P_4) were assayed by RIA kits (Immunotech, France). Pregnancy Diagnosis was carried out at day 75-90 of breeding in non return buffaloes and conception rate was assessed by dividing no. of animals Pregnant by total no. of Inseminations (expressed as %).

The mean pre and post AI pulse rate recorded in control and treatment groups were 61.44±1.29, 61.84±1.23 /min and 62.81±1.22, 65.63±1.13 /min respectively.

The mean pre and post AI respiration rate recorded in control and treatment groups were 10.00±0.45, 10.24±0.42 /min 10.44±0.45/min, 12.29±0.44/min respectively. Comparison of pre and post AI PR and RR revealed that there was a significant increase in the treatment group. However, in the control group, neither the PR nor the RR was significantly affected indicating that AI did not affect these vital parameters *per se*. The values were comparable in the control and treatment buffaloes before A.I., however they were differing significantly post-AI.

There was no significant effect of time on the LH level either in the control or treatment group. Similarly, the interaction between the clitoral stimulation treatment and time was not significant. Overall, there was a significant increase in the mean LH level in treatment group (6.405±2.64 ng/ml) as compared to control (2.37± 0.838 ng/ml) irrespective of the sampling time. This is due to the fact at every time point tested except 4^{th} & 6^{th} hr, treatment group had higher values of LH than that of control. Peak LH conc. of animals in the treatment group was higher and the peaks occurred within 5 hours of stimulation presumably resulting in the better synchrony among insemination-ovulation-fertilization.

There was no significant difference between E_2 concentrations of control and treatment groups on different sampling times, indicating neither clitoral stimulation nor sampling time significantly affected the levels of estradiol in the experimental buffalo.

Serum P_4 concentration in control and treatment group at 0, 6,12 and 18 days ranged between 0.69±0.10 - 4.04±0.61 and 0.823±0.10 - 6.75±1.04 ng/ml respectively. Lowest values were recorded on the day of estrus in both the groups. No significant difference was observed between both the groups on different sampling days, except at day 18 when significantly higher levels of serum P_4 were detected in treatment group as compared to control (6.75±1.04 Vs 4.04±0.61 ng/ml), which was a reflection of more no. of animals getting pregnant in treatment group.

The overall service per conception in treatment and control buffaloes was 1.48 and 2.88, respectively. Just 1.48 inseminations were required for a conception in parous treatment group while it was 3.09 in control. In heifers, 1.5 inseminations were made to make an animal conceive in treatment group as compared to 2.4 inseminations in control.

Experimental buffaloes were divided in parous and heifer categories, to examine the effect of parity. The CR in parous buffaloes in treatment group (67.65%) was found to be significantly higher as compared to control (32.35%). The CR in treated heifers (66.67%) was also higher than control (41.67%) but statistical significance could not be obtained. However, the pooled conception rate in the treatment group (67.39%) was found to be significantly higher as compared to control (34.78%). The odds of getting pregnant after clitoral stimulation in parous buffalo was 4.37 times high as that of control. The odds of getting pregnant after clitoral stimulation in heifers was 2.80 times high as that of control. The overall odds of conception in a buffalo that received clitoral massage at the time of AI was 3.875 times high as that of control.

Study indicates that clitoral stimulation at the time of AI in buffaloes has a significant positive effect on endogenous LH release, presumably influencing the ovulation time and thereby augmenting CR. Hence it can be recommended that clitoral stimulation at the time of AI may be adopted as a package of practice in bovines, more particularly in buffaloes as the national figure of CR from AI in field condition needs to be enhanced to exploit the fullest potential of this versatile species.

Mini Abstract

MINI ABSTRACT

Buffalo has a higher incidence of delayed ovulation than cattle and conception rate to AI is generally lower. The present study was planned with the objectives to study the effect of clitoral stimulation after A.I. on physiological parameters, reproductive hormone profiles (luteinizing hormone, estradiol and progesterone) and conception rate in buffaloes. The study was conducted on 92 apparently healthy female Murrah buffaloes maintained at the Cattle and Buffalo Farm, LPM section, IVRI, during the breeding season. Estrus was identified on the basis of teaser bull parading twice daily and visual observation of estrus signs. The buffaloes were first divided in parous (n=68) and heifer (n=24) categories, to examine the effect of parity. Further, parous animals were subdivided into treatment (n=34) and control (n=34) groups. Similarly heifers were subdivided into treatment (n=12) and control (n=12) groups. Animals in treatment group were given thirty seconds of clitoral stimulation (massage) after AI while no such stimulation was given in control animals. Physiological response in experimental animals in terms of pulse rate (PR) and respiration rates (RR) were recorded. Eight samples of blood were collected for treatment animals (n=3) against control (n=3), at an hourly interval for LH and estradiol estimation. For P_4 hormone estimation the blood was collected on day of estrus, 6^{th},12^{th} and 18^{th} post AI (n=6/ group). Serum LH concentration were determined using Bovine LH ELISA test kit while ovarian hormone (E_2 and P_4) were assayed by RIA kits. Comparison of PR and RR pre and post AI revealed that there was a significant increase in the treated group. However, in the control group, neither the PR nor the RR was significantly affected indicating that AI did not affect these vital parameters *per se*. Overall, there was a significant increase in the LH level in treatment group (6.405±2.64 ng/ml) against control (2.37± 0.838 ng/ml) irrespective of the sampling time. Peak LH conc. of animals in the treatment group was higher and the peaks occurred within 5 hours of stimulation. Neither clitoral stimulation nor sampling time significantly affected the levels of estradiol. On day 18, Significant increase in serum P_4 treatment group was observed with respect to animals in control which was a reflection of more no. of animals getting pregnant in treatment group. The CR in the treatment group (67.39%) was found to be significantly (P<0.01) higher as compared to control (34.78%). CR in treated parous buffaloes (67.65%) was found to be significantly higher as compared to control (32.35%). Though, the CR in treated heifers (66.67%) was higher than control (41.67%) but not significant. The overall odds of conception in a buffalo that received clitoral massage at the time of AI was 3.875 times high as that of control.

लघु सारांश

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भैंसों में गाय की तुलना में देरी से डिंबक्षेपण की समस्या अधिक पाई जाती है एवं कृत्रिम गर्भाधान करने पर गर्भधारण दर प्रायः कम रहती है। वर्तमान अध्ययन का उद्देश्य भैंसों में कृत्रिम गर्भाधान के समय क्लाइटोरल (भग्नशिश्निका) उद्दीपन का प्रभाव दैहिकी मानकों, जनन सम्बन्धी हारमोन सान्द्रण {ल्यूटीनाजिंग हारमोन (एल.एच.) ईस्ट्राडियाल एवं प्रोजेस्टेरान} एवं गर्भधारण दर पर ज्ञात करना था। भारतीय पशुचिकित्सा अनुसंधान के पशुधन उत्पादन प्रबंधन अनुभाग के अन्तर्गत गाय एवं भैंस प्रक्षेत्र की 92 स्वस्थ्य मुर्रा भैंसो पर यह अध्ययन प्रजनन काल में किया गया। भैंसों में मद की जाँच टीजर सा ॅंड द्वारा एवं लक्षण देखकर दिन में दो बार की गई। सर्वप्रथम भैंसों को प्रसवित (एन=68) एवं पड़ियों में विभाजित किया गया तत्पश्चात प्रसवित पशुओं को उपचार (एन=34) एवं नियन्त्रण (एन=34) समूहों में बा ॅंटा गया। इसी प्रकार पड़ियों को उपचार (एन=12) एवं नियंत्रण (एन=12) समूहों में बा ॅंटा गया। उपचार समूह में पशुओं में कृत्रिम गर्भाधान के पश्चात् 30 सेकेण्ड तक क्लाटोरल उद्दीपन किया गया। जबकि नियंत्रण समूह में पशुओं को उद्दीपित नहीं किया गया। पशुओं में नाडी एवं श्वसन दर ज्ञात की गई। उपचार (एन=3) एवं नियंत्रण (एन=3) समूहों में 1 घण्टे के अन्तराल पर रक्त के आठ नमूने एल.एच.एवं ईस्ट्राडियाल सान्द्रण ज्ञात करने हेतु एकत्रित किये गये। प्रोजेस्टेरान सान्द्रण ज्ञात करने हेतु रक्त के नमूने मद के दिन एवं 6, 12, 18 (एन-6 प्रति समूह) दिन पर एकत्रित किये गये एल.एच. सान्द्रण बोवाइन एल. एच. एलाइजा टेस्ट किट एवं डिम्बाशयी हारमोनों का स्तर आर. आई ए. किट द्वारा मापा बाया। श्वसन एवं नाड़ी दर उपचारित समूह में कृत्रिम गर्भाधान के पश्चात् सार्थक रूप से बढ़ी हुई थी। नियंत्रण समूह में ऐसी कोई वृद्धि ज्ञात नहीं हुई। एल.एच का सानद्रण उपचारित समूह में (6.405±2.64 नैनोग्राम प्रति मिली) नियंत्रण समूह (2.37±0.83 नैनोग्राम मिली) अपेक्षा सार्थक रूप से अधिक दर्ज किया गया। उपचारित समूह में एल.एच. का चरम सान्द्रण अधिक एवं उद्दीपन के 5 घंटे के भीतर पाया गया। क्लाइटोरल उद्दीपन एवं नमूने रक्त करने के समय का ईस्ट्राडियाल के सान्द्रण पर कोई सार्थक प्रभाव नहीं था। रक्त में प्रोजेस्टेरान का सान्द्रण उपचारित समूह में 18 वें दिन सार्थक रूप से बढ़ा हुआ था क्योंकि इस समूह में अधिकांश पशु गर्भित हो गये थे। उपचारित समूह में गर्भधारण दर (67.37%) नियंत्रण समूह (34.78%) की अपेक्षा सार्थक रूप से अधिक थी। प्रसवित भैंसों में यह दर उपचार समूह (67.65%) में नियंत्रण समूह (32.35%) की अपेक्षा सार्थक रूप से अधिक ज्ञात हुई। पड़ियों में गर्भधारण दर उपचार समूह (66.67%) में नियंत्रण समूह (41.67%) की अपेक्षा अधिक किन्तु सार्थक रूप से भिन्न नहीं थी।

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