Effects of Pre-Treatment with GnRH on the Efficiency of Superstimulatory Protocol in Water Buffalo (Bubalus bubalis)

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The multiple ovulation embryo transfer technique (MOET) in buffalo has yielded poor superovulation response due to presence of large/dominant follicle(s) at the start of superstimulatory treatment. The aim of present study was to evaluate the effects of pretreatment with gonadotropin on number of total as well as viable and transferrable embryos recovered during MOET programme in Murrah buffaloes. Buffaloes (n=27) enrolled into two treatment (groups A and B, n=9 in each group) and one control (group C, n=9) groups were administered with 600 mg of follicle stimulating hormone (FSH) in 10 decreasing doses at 12 hourly interval for five days. Additionally, buffaloes were administered with a single dose of GnRH in group A (10 µg) and group B (06 µg) as a pre-treatment on 2.5 day prior to the start of FSH. Two doses of prostaglandin (500 µg) were administered with the 7th and 8th dose of FSH in all the buffaloes. Embryos were collected non-surgically on day 5.5 post-insemination. Number of total, transferrable and the non-transferrable number of embryos recovered were recorded in the 3 groups. Total as well as the viable transferrable number of embryos recovered were significantly higher (P<0.05) in the buffaloes of group B compared to the control (3.0 vs 1.33 and 2.33 vs 1.0, respectively). In all the three groups, about 75% of embryos recovered were of transferrable grade. The results of study indicated that administration of GnRH as a pre-treatment in superstimulatory treatment improved the MOET efficiency in buffalo.

KEY WORDS buffalo, embryo, follicle stimulating hormone, GnRH.

INTRODUCTION

Livestock sector is an integral component of agriculture farming system and have a pivotal role in the rural economy of India. The water buffalo is a premier dairy animal in India and many other south Asian countries. Poor reproductive performance of buffaloes due to delayed puberty, silent estrus, poor conception rates, long postpartum interval etc. causes heavy economic losses to the livestock farmers. This led to diminishing interest of the farmers in buffalo husbandry as indicated by declining growth rate of buffalo population during the last decade. Amongst the various reproductive biotechniques multiple ovulation embryo transfer technology (MOET) could be a viable option to augment the need of faster genetic gain for making buffalo farming profitable. The suitable superstimulatory and embryo flushing protocols for buffaloes are ideally different from cattle due to differences in species, ovarian follicular population and dynamics; and developmental rate and transport of early embryo. Generally, a greater dose of FSH
(600 mg) in decreasing schedule for buffaloes for superstimulation and the embryo flushing on day 5.5 after artificial insemination (AI) was recommended (Misra et al. 1998). Furthermore, the number of ovulations, total and viable transferrable embryos recovered were lesser in buffalo compared to cattle (Baruselli et al. 2018). The aforesaid parameters are largely affected by the recruitment of follicular wave, presence of dominant follicle and progesterone milieu present at the start of superstimulatory treatment (Misra and Tyagi, 2007). Presence of dominant follicle at initiation of ovarian super-stimulation have a negative (Kohram and Poorhamdollah, 2012), whereas higher progesterone concentration have a positive effect (Ravi et al. 2011) on the outcomes of superstimulation. During the last decades, a few approaches were proposed to manipulate the ovarian follicular dynamics to circumvent the effect of dominant follicle so as to improve the success of MOET. Ablation of the dominant follicle (Leonardo et al. 2019), manipulation of the estrus cycle (by progesterone and oestradiol preparations; (Bulbul et al. 2010; Bulbul et al. 2013) and pre-treatment with gonadotropin releasing hormone (GnRH) (Sato et al. 2005) have been successfully used to manipulate and induce the emergence of a new follicular wave to optimize the superovulatory outcomes in cattle. Administration of GnRH causes ovulation/luteinization of the dominant follicle (Pursley et al. 1995) and 2-3 days later emergence of a new follicular wave (Naseer et al. 2012). The applicability of this aspect has not been evaluated during superstimulation in buffaloes. Moreover, ovary on the 7 or 8 day after end of estrus would probably have a large dominant follicle responsive to exogenous gonadotropin (Baruselli et al. 2013), irrespective of buffalo having whether 2-wave or 3-wave follicular dynamics. We hypothesised that GnRH treatment on 7.5 day post-estrus would alleviate the negative effect of dominant follicle and improve the superstimulatory attributes when we start superovulatory treatment 2.5 day later to it. Accordingly, the present study was conducted to evaluate the effect of pre-treatment with two different doses of GnRH on the total and viable embryo recovery in buffalo.

**MATERIALS AND METHODS**

**Animal selection**

Twenty seven Murrah buffaloes (Bubalus bubalis) maintained at the institutional dairy farm were assigned into this study. Buffalo enrolled into the study were in the second to fourth parity, lactating, had a mean age of 6.2 ± 1.4 years, weighing 450-560 kg, minimum 70 days post-partum and a body condition score (BCS) of 3.5–4.0 (5-point scale; Alapati et al. 2010). Healthy normal cyclic buffaloes with healthy genitalia and patent cervix during diestrus were selected based on the gynaecological and trans-rectal ultra-sonographic examinations. Animals were provided with *ad libitum* drinking water and daily rations comprising of fresh green fodder, straws and concentrates. The experimental protocol was approved by the institutional animal ethics committee.

**Experimental design**

Buffaloes were randomly selected for two treatment groups (group A and B) and one control group (group C). Each treatment group (*n=*9) was subjected to a different dose of GnRH as a pre-treatment strategy. Buffaloes were observed for spontaneous estrus or those with palpable mature corpus luteum were induced to estrus with prostaglandins (PGF2α; 500 µg; cloprostenol sodium, intramuscularly). The estrus was detected at 12 h interval following 48 h after PGF2α injection by close observation of behavioral external signs (bellowing, mounting, frequent micturition, cervico-vaginal mucus discharge, swollen and edematous vulva) and was confirmed by per-rectal examination of uterine tonicity, cervico-vaginal discharge and its arborization. A schematic schedule of protocols used for *in vivo* embryo production is depicted in Figure 1. On seventh day after the end of estrus, pretreatment with gonadotropin (GnRH) was administered intramuscularly in two groups with different doses. i.e. 10 µg GnRH in Group A (*n=*9) and 06 µg GnRH in Group B (*n=*9), while group C (control group) received an injection 2 mL of distilled water as a placebo. Superstimulatory treatment was started in buffaloes of all the three groups on day 10 after the end of estrus. All buffaloes were injected with 600 mg follicle stimulating hormone intramuscularly (FSH; Folltropin-V®, Bioniche Animal Health Inc., Canada) in 10 divided decreasing doses (80:80, 70:70, 60:60, 50:50, 40:40 mg) at 12 h interval (morning and evening) over the next five day period. Prostaglandin (cloprostenol sodium, 500 µg intramuscularly) was given with seventh and eighth dose of FSH for luteolysis in order to induce estrus 36-48 h later. Fixed timed artificial inseminations (AI) were done thrice at 12 h interval starting from 36 h of the first prostaglandin injection using good quality frozen-thawed semen (with at least 10 million motile spermatozoa) of an elite Murrah buffalo bull.

**Embryo flushing and evaluation**

Superovulatory response in terms of number of corpus lutea (CL) formed was assessed manually prior to embryo flushing. Flushing of embryos from the uterus on 5.5 day post-first AI was done by non-surgical method under mild sedation (xylazine hydrochloride 5-10 mg) and epidural anesthesia (lignocaine hydrochloride 40-100 mg) using 18 G silicon catheter as described previously (Singhal, 2010). In brief, each uterine horn was flushed using about 500 ml of flushing medium (Dulbecco’s phosphate buffer saline) containing 0.1% bovine serum albumin.
After completing the process of embryo flushing, antibiotic suspension (30 mL of 4% gentamicin sulphate solution) was infused into the uterus as a precautionary measure to protect against uterine infection. A luteolytic agent was administered intramuscularly to bring the animal in estrus again. Flushed out media was collected in an Emcon filter and transferred into petridish (90×10 mm; Nunc, Thermo Fisher Scientific, USA).

A thorough searching for embryos was undertaken using stereozoom microscope (10-50×magnification) and the embryos were categorized as of viable transferrable and non-transferrable quality according to standard classification (Phillips and Jahnke, 2016).

Statistical analyses
Statistical software (SPSS, 2011) was used for the making the statistical comparisons of the data. Difference between means was compared using students ‘t’ test. The percent of transferrable embryos between the groups were compared using Chi-square test. P < 0.05 were considered to be statistically significant. The findings obtained in this study are presented as mean values and percentages. All mean values are expressed as the mean ± standard error of mean (Table 1).

RESULTS AND DISCUSSION
The present study evaluated the effects of additional pretreatment with GnRH on the outcomes of superstimulation during MOET in buffaloes. Two treatment groups i.e. groups A and B were treated with 10 and 06 µg GnRH, respectively whereas group C was administered with 2 mL DW to serve as control. Variables pertaining to superovulatory response viz. (mean number of corpora lutea, mean number of total embryos recovered, mean number of transferrable embryos, mean number of non-transferrable embryos, percentage of transferrable embryos) obtained in all the groups are depicted in Table 1.

Superovulatory response
Superovulatory response (SOR) was evaluated by counting the total number of corpus luteum (CL) palpable per-rectally over both the ovaries. Both the experimental groups treated with GnRH showed higher SOR than control and group B (GnRH pretreatment @ 06 µg) differs significantly (P<0.05) with control (Table 1).

Total embryos recovery
The embryo recovery rate was calculated as = total number of ova and embryos recovered/number of CL detected × 100. A total of 27 uterine flushings (nine in each group) for embryo collection were executed in the study and a total of 58 embryos were recovered. The average total number of embryos recovered per group were 2.11 ± 0.61, 3.0 ± 0.71 and 1.33 ± 0.33, respectively in group A, B and C. GnRH pretreatment (06 µg IM) showed significantly higher (P<0.05) total embryo recovery in comparison to control. In the similar pattern, buffaloes of group B have highest transferrable embryos per flushing (2.33±0.64) which was significantly higher (P<0.05) than control but didn’t differ significantly from other GnRH pretreated group A. Pooled data of all groups revealed that the mean total and average viable transferrable embryos were 2.15 ± 0.34 and 1.63 ± 0.30, respectively per animal.

Recovery of viable transferrable embryos
In the study, percentage of transferrable embryos recovered from total embryos was 73.7, 77.8 and 75% for group A, B and C, respectively. Percent transferrable recovery and non-transferrable embryos per flushing did not differ significantly (P<0.05) among the three experimental groups.

Recovery of poor quality embryos
The current study observed non-transferrable or poor quality embryos were 0.55 ± 0.18, 0.66 ± 0.23 and 0.33 ± 0.17 in group A, B and C, respectively. These values were statistically similar in the three experimental groups.

Figure 1 Time line for the experimental groups in superstimulatory protocol
FSH: follicle stimulating hormone (in mg) and PG: prostaglandin and DW: distilled water
Recovery of poor quality embryo must be low for improved efficiency of MOET as indicated in this study compared to other reports, which may be caused by various genetic or non-genetic factors involved in different studies (Misra and Tyagi, 2007).

In this study, we demonstrated the effects of pretreatment by GnRH on superstimulatory variables in water buffaloes. In the current study, the average recovery of 2.15 embryos per flushing (58/27) was recorded and is higher than results of other study during presence of dominant follicle but is equivalent if superstimulation was done using PMSG (Follikon, 3000 IU) in absence of dominant follicle (Heleil and El-Deeb, 2010). The total number of embryos recovered per animal in present study is in agreement with the equivalent recovery of average 1-3 embryo reported earlier using 400 mg Follitropin (Redhead et al., 2018), but lower than about four embryos obtained per animal in another study (Hesheng et al. 2006). Neglia et al. (2010) showed similar total recovery of 2.3-2.8 embryos per buffalo using PRID and 500 IU of FSH while very poor recovery has been reported in heifers (Kandil et al. 2012). These discrepancies in embryo recovery might due to differences in weather conditions (Phogat et al., 2016), age, parity and lactational status of donor (Misra and Tyagi, 2007).

The present study observed significantly (P<0.05) greater mean number of corpora lutea in treatment group B compared to control (8.4±0.72 vs. 6.17±0.45). On same pattern, mean total embryo recovery in group B was significantly (P<0.05) higher (3.0±0.71) than mean embryo recovered (1.33±0.33) from control experimental group (Table 1). In current study, embryo recovery rate in relation to number of ovulation(s) is lesser which could be attributed to the inability of fimbria to trap ova from enlarged superovulatory ovary, difficulty in locating hatched blastocytes and or premature entry of ova/embryos into the uterus (Karaivanov et al. 1990; Ullah et al. 1992). The altered biochemical milieu during the superovulation might also result in impaired ovulation (Angela et al. 2018) and the oviductal transport (Carvalho et al. 2006).

Although much higher than many earlier reports, the percentage of viable transferrable grade embryos yielded (about 75%) in the present study is similar in all the 3 groups. Similarly, 50-80% of embryos recovered were of transferrable quality with or without GnRH treatment during estrus (Techakumphu et al. 2001). The varied viable embryo recovery rate from 32.6% to 84.4% in superovulated buffaloes inseminated using the frozen-thawed semen of five different bulls have also been reported (Misra et al. 1999). Variation in agro-climatic zones or the use of semen of a single elite bull might be the cause of reasonably greater proportion of transferrable grade embryos obtained in the present study. However, the overall yield of average viable and transferrable embryos (1.63; 44/27) per buffalo from the 27 flushings recorded in the present study is very similar to earlier observation (1.62; Kandil et al. 2012). These results are in concurrence with several other reports of an average yield of 1-3 viable embryos per flushing (Baruselli et al. 2013). In all the three groups, we noticed that the quality of embryos was better when embryos were recovered in multiple number (>1) rather than the recovery of single embryo in a uterine flushing. This effect may be due to cell to cell communication signals which promote mutual growth and development of the developing embryos, leading to improved quality in the cohort. Correspondingly, better in vitro developmental rates of oocytes and early embryos have been observed when cultured in groups compared to the individual (Salvador et al. 2011).

GnRH has important role in regulation of the recruitment, selection, dominance and ovulation of follicles. These events have been induced with exogenous gonadotropins in gonadotropin deprived sheep (Driancourt et al. 1987). There is limited information available in the literature regarding utilization of GnRH pretreatment prior to the superstimulatory protocol in buffalo. Use of the GnRH obviates the effect of dominant follicle either by causing its ovulation or leutinization during estrus synchronization in bovine (Mohammadi et al. 2019) and during MOET in cattle (Sato et al. 2005).

### Table 1

Superstimulatory response in buffaloes pretreated with different doses of GnRH (Mean±SEM)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group A (GnRH, 10 µg)</th>
<th>Group B (GnRH, 06 µg)</th>
<th>Group C (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean number of corpora lutea per donor</td>
<td>7.0±0.65</td>
<td>8.4±0.72</td>
<td>6.17±0.45</td>
</tr>
<tr>
<td>Mean number of total embryo(s) recovered per flushing</td>
<td>2.11±0.61</td>
<td>3.0±0.71</td>
<td>1.33±0.33</td>
</tr>
<tr>
<td>Mean number of transferrable embryo(s) per flushing</td>
<td>1.55±0.53</td>
<td>2.33±0.64</td>
<td>1.0±0.29</td>
</tr>
<tr>
<td>Mean number of non-transferrable embryo(s) per flushing</td>
<td>0.55±0.18</td>
<td>0.66±0.23</td>
<td>0.33±0.17</td>
</tr>
<tr>
<td>Percent of transferrable embryo(s)</td>
<td>73.68%</td>
<td>77.78%</td>
<td>75.0%</td>
</tr>
</tbody>
</table>

The means within the same row with at least one common letter, do not have significant difference (P>0.05). SEM: standard error of the means.
Therefore, GnRH would increase the progesterone concentration by increasing luteal tissue and may also recruit a new follicular wave within two to three days. It was indicated that administration of GnRH induced the emergence of a new follicular wave within 2-3 days after the induced ovulation of dominant follicle in cattle (Macmillan and Thatcher, 1991) and in about 60% of buffalo (Baruselli et al. 2013). Present study observed, the mean CL number were greater in both the experimental groups compared to the control group, however, it didn’t vary significantly among GnRH treated groups and was numerically higher with lower dose treatment. In the current study, FSH treatment given on 2.5 days after the GnRH might have coincided with a newly recruited follicular wave and lead to better superovulatory outcomes both in terms of embryo recovery rate as observed in the treatment groups compared to control group. In accordance, a lesser dose of GnRH given 2.5 days prior to superstimulation showed improved superovulatory outcomes in cattle (Sato et al. 2005). Similarly, increased ovulation rate and embryo recovery were observed in absence of the dominant follicle in cattle (Bungartz and Niemann, 1993) and buffalo (Honparkhe et al. 2014). Contrarily, there was no difference in superovulatory response in cows when the superstimulatory treatment was initiated in presence or absence of dominant follicle (Wilson et al. 1990). Moreover, endogenous release of LH is dependent on dose of GnRH administered exogenously (Veldhuis et al. 1989). In the present study, lower dose of GnRH (06 µg) improved the superovulatory outcomes because buffaloes are more sensitive to exogenous hormonal therapy than cattle (Nanda et al. 2003). This finding is supported by the observation that a higher dose of GnRH agonist reduced sensitivity and number of receptors of GnRH on hypothalamus (Ulker et al. 2001), which in turn suppresses the endogenous secretion of gonadotropins.

**CONCLUSION**

In conclusion, the study indicated that the application of pretreatment with GnRH on 2.5 days prior to start of the superstimulatory protocol improved the yield of total and viable grade embryos. Hence, further elaborate experiments are warranted to establish the beneficial effect of GnRH pretreatment to replace the conventional superovulatory protocols for improving efficiency of MOET in buffaloes.

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**REFERENCES**


