Comparison of follicular dynamics, superovulatory response, and embryo recovery between estradiol based and conventional superstimulation protocol in buffaloes (Bubalus bubalis)

Narinder Singh1, G. S. Dhaliwal2, V. S. Malik1, D. Dadarwal3, M. Honparkhe1, S. Singhal1 and P. S. Brar1

1. Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India; 2. Department of Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India; 3. Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada.

Abstract

Aim: To evaluate the follicular dynamics, superovulatory response, and embryo recovery following superstimulatory treatment initiated at estradiol-17β induced follicular wave emergence and its comparison with conventional superstimulatory protocol in buffaloes.

Materials and Methods: Six normal cycling pluriparous buffaloes, lactating, 90-180 days post-partum, and weighing between 500 and 660 kg were superstimulated twice with a withdrawal period of 35 days in between two treatments. In superstimulation protocol-1 (estradiol group) buffaloes were administered estradiol-17β (2 mg, i.m.) and eazibreed controlled internal drug release (CIDR) was inserted intravaginally (day=0) at the random stage of the estrous cycle. On the day 4, buffaloes were superstimulated using follicle stimulating hormone (FSH) 400 mg, divided into 10 tapering doses given at 12 hourly intervals. Prostaglandin F2α analogs (PGF2α) was administered at day 7.5 and day 8, and CIDR was removed with the second PGF2α injection. In superstimulation protocol - 2 (conventional group) buffaloes were superstimulated on the 10th day of the estrous cycle with same FSH dose regimen and similar timings for PGF2α injections. In both groups, half of the buffaloes were treated with luteinizing hormone (LH) 25 mg and other half with 100 ug buserelin; gonadotrophin releasing hormone (GnRH) analog at 12 h after the end of FSH treatment. All buffaloes in both protocols were inseminated twice at 12 and 24 h of LH/GnRH treatment. Daily ultrasonography was performed to record the size and number of follicles and superovulatory response.

Results: Significantly higher number of small follicles (<8 mm) was present at the time of initiation of superstimulatory treatment in the estradiol group compared to the conventional group (12.5±0.80 vs. 7.3±1.21, respectively, p=0.019), however, the number of ovulatory size follicles (≥8 mm) did not differ significantly between the respective groups (15.5±1.24 vs. 12.2±1.30; p=0.054). Total embryos and transferable embryos recovered were non-significantly higher in the estradiol group compared to the conventional group (5.8±0.86 vs. 4.6±1.16, p=0.328, and 3.67±0.93 vs. 2.67±0.68, p=0.437, respectively). The significant higher proportion of transferable embryos were recovered in buffaloes treated with LH compared to GnRH (73.3% vs. 48.5%; p=0.044).

Conclusion: The average number of ovulatory size follicles (>8 mm), corpora lutea, and transferable embryos was higher in buffaloes superstimulated at estradiol-induced follicular wave compared to the conventional protocol: Further the percentage of transferable embryos was significantly higher in buffaloes administered with LH compared to GnRH.

Keywords: buffalo, embryo, estradiol-17β, superstimulation, ovulation rate.

Introduction

Buffalo is the mainstay of Indian dairy industry contributing more than 50% of milk to national milk pool which is much higher than the contribution of almost double the population of cattle [1]. However, the productivity of buffalo is low and is significantly hampered by various reproductive factors namely late attainment of puberty, poor estrus expression, seasonality, and post-partum anestrus. Multiple ovulation and embryo transfer (MOET) involving superstimulation and in vivo embryo collection have the potential to increase the contribution of superior females buffaloes to the gene pool of the population and it has been successfully used for faster dissemination of superior germplasm through the production of breeding sires from superior females in cattle [2,3]. In buffaloes, the application of MOET is limited due to variable superovulatory response and low yield of (<1) transferable embryos [4,5]. Similar to cattle, Misra [6] reported that over 26% superstimulated buffaloes produced ≥6 embryos per collection and in vivo embryo production could be a viable option in buffaloes on the identification of good donors.

So far conventional superstimulatory protocols have been used in buffaloes involving the initiation
of superstimulatory treatment during mid-cycle (8-12th day), to coincide with the approximate time of emergence of the second follicular wave. It has been observed that asynchrony of even 1 day between wave emergence and initiation of superstimulatory treatment may significantly reduce the superovulatory response [7]. Therefore, accurate estrus detection followed by initiation of superstimulatory treatment coinciding with the emergence of the second follicular wave is the key factors to achieve optimal results. Detection of estrus is difficult in buffaloes and the emergence of second follicular wave may also differ depending upon the estrous cycle being two- or three-wave affecting the initiation of superstimulatory treatment at emergence of second follicular wave in conventional protocols. Further, the presence of dominant follicle is reported to have an inhibitory effect on superstimulation that means nearly 80% period of the estrous cycle is not conducive to initiate superstimulatory treatment. Beg et al. [8] also pointed out that the standardizing the superovulatory schemes in the middle of the estrus cycle are more difficult in buffaloes compared to cattle. To obviate these problems, an alternative approach could be to develop protocols which eliminate the need of estrus detection and necessary waiting until mid-cycle for initiating the superstimulatory treatment and permit fixed timed insemination of donors. In cattle, this has been successfully achieved following the induction of new follicular wave by removing dominant follicle through mechanical (follicle ablation) or hormonal approaches (reviewed in [9]). In buffaloes, few attempts have been made to apply such protocols with limited success [10]. However, no comparative study between initiation of superstimulatory protocol on exogenously controlled follicular wave emergence and conventional protocol is available in buffaloes. The emergence of follicular wave can be controlled in buffaloes through the administration of 1.5 mg estradiol-17β at a random stage of the estrous cycle [11] which could facilitate recruitment of a pool of growing follicles of uniform size on superstimulation. Therefore, the study was designed to assess the impact of initiating superstimulatory treatment at the beginning of estradiol-17β-induced follicular wave emergence on follicular dynamics, superovulatory response, and viable embryo recovery and its comparison with the conventional protocol in buffaloes.

Materials and Methods
Ethical approval
The present study being a part of a larger study for doctorate thesis, approval had been obtained from the Institutional Animal Ethics Committee. The embryo collection was done as per standard procedure without harming the animals.

Treatment groups
Six normal cycling healthy buffaloes, with average body weight 500 - 660 kg, 90-180 days post-partum, superstimulated twice with a withdrawal period of 35 days in between two treatments were used for the study.

In Group I (estradiol group, n=6): Buffaloes were administered estradiol-17β (2 mg, i.m.) along with insertion of eazibreed controlled internal drug release (CIDR) (designated as day=0), the superstimulatory treatment was initiated on the 4th day (day of follicular wave emergence) using follicle stimulating hormone (FSH) 400 mg NIH-FSH-P1, given daily intramuscularly in divided, tapering doses (60, 60, 50, 50, 40, 40, 30, 30, 20, 20 mg), over a period of 5 days. All the buffaloes were given two injections of prostaglandin F2α analog (PGF2α) cloprostenol 500 μg at 84 and 96 h after the start of superstimulatory treatment and CIDR was removed at the time of second prostaglandin injection.

Group II conventional superstimulation protocol: Buffaloes (n=6) were superstimulated as per the conventional protocol by initiating the superstimulatory treatment on the 10th day of induced estrus using same dose regimen of FSH as above. Similarly, buffaloes were given two injections of PGF2α (500 μg cloprostenol; i/m) at 84 and 96 h after the start of superstimulatory treatment.

In both Groups I and II, half of the buffaloes were administered 25 mg luteinizing hormone (LH), and half were administered 100 ug buserelin (gonadotrophin releasing hormone (GnRH) analog) at 12 h after the end of superstimulatory treatment. The fixed time insemination was done at 12 and 24 h of LH/GnRH treatment.

Observations
Ultrasonography was carried out daily using ultrasound machine (Mindray, China) equipped with B-mode linear array trans-rectal probe (7.5 MHz) from the start of experiment till the day of embryo recovery to record the size and the number of follicles followed with number of ovulations and luteal dynamics. Embryos were collected by flushing the uterus of the donor animals non-surgically on day 5.5 using two-way Worrlien catheter using flushing media - Dulbeco’s phosphate buffered saline supplemented with 0.4% bovine serum albumin and antibiotics at standard rates. Embryos were searched and evaluated morphologically using zoom stereomicroscope and were graded as per the manual of International Embryo Transfer Society [12].

Statistical analysis
The data are presented as means and standard errors for all variables. After confirming the normality of data and homogeneity of variance, Student’s t-test (two-tailed) was applied to compare mean values of treatments in SPSS-16 Statistical program. A p=0.05 was considered as significant.

Results
All the buffaloes responded to superstimulatory treatment by developing multiple corpora lutea (CLs) on the ovaries. The results are presented in Table-1.
Follicular dynamics and embryo production in buffalo’s superstimulated using the estradiol based and the conventional superstimulation protocol in buffaloes.

| Parameters                                                                 | Estradiol based protocol (n=6) | Conventional protocol (n=6) |
|----------------------------------------------------------------------------|---------------------------------|-----------------------------|
| At start of superstimulation protocol                                       |                                 |                             |
| Number of follicles < 8 mm                                                 | 12.50±0.80                      | 7.33±1.21                   |
| Number of follicles ≥8 mm                                                  | 0.17±0.20                       | 0.83±0.32                   |
| On the day of estrus                                                       |                                 |                             |
| Number of follicles < 8 mm                                                 | 2.67±1.12                       | 3.50±0.75                   |
| Number of follicles ≥8 mm                                                  | 15.50±1.24                      | 12.17±1.30                  |
| Total number of CLs (day 5 post AI)                                        | 12.17±1.36                      | 10.33±0.63                  |
| Ovulation rate (number of ovulatory size follicles/number of CLs)          | 78.49                           | 84.9                        |
| Number of anovulatory follicles≥8 mm (day 5 post AI)                       | 2.1±1.75                        | 1.0±0.60                    |
| Number of embryos+ova recovered                                            | 5.83±0.86                       | 4.67±1.16                   |
| Recovery rate (number of embryo+ova recovered/number of CLs)              | 47.95                           | 45.16                       |
| Transferable embryos                                                      | 3.67±0.93                       | 2.67±0.68                   |
| Percent transferable embryos                                               | 62.86                           | 57.14                       |

Values marked with superscript (*) in a row differ significantly at 5% level, CLs=Corpora lutea, AI=Artificial insemination

Table-2: Effect of LH and GnRH on ovulation, embryo recovery, and the number of transferable embryos in buffaloes.

| Parameters                                                                 | LH (n=6) | GnRH (n=6) |
|----------------------------------------------------------------------------|----------|------------|
| Number of follicles on day of estrus                                       |          |            |
| Number of follicles < 8 mm                                                 | 3.17±0.91 | 3.0±0.80   |
| Number of follicles ≥8 mm                                                  | 14.50±0.92 | 13.17±1.68 |
| Number of CLs (day 5 post AI)                                              | 12.0±1.21 | 10.50±0.55 |
| Ovulation rate (%)                                                         | 82.7     | 79.7       |
| Number of anovulatory follicles≥8 mm (day 5 post AI)                       | 1.0±0.63  | 2.0±1.62   |
| Number of embryos+ova recovered                                            | 5±1.24   | 5.50±0.93  |
| Recovery rate (%)                                                          | 41.7     | 52.4       |
| Number of transferable embryos                                             | 3.67±0.76 | 2.67±0.73  |
| Percent transferable embryos                                               | 73.3*    | 48.5*      |

Values marked with superscript (*) in a row differ significantly at 5% level, CLs=Corpora lutea, LH=Luteinizing hormone, GnRH=Gonadotropin releasing hormone, AI=Artificial insemination

Significantly higher number of small follicles (<8 mm) was present at the time of initiation of superstimulatory treatment in the estradiol group compared to the conventional group (12.5±0.80 vs. 7.3±1.21, respectively, p=0.019), however, during estrus the number of ovulatory size follicles (≥8 mm) did not differ significantly between the respective groups (15.5±1.24 vs. 12.2±1.30; p=0.054). The average number of follicles ≥8 mm size were higher at the time of initiation of superstimulatory treatment during mid-cycle in the conventional group compared to the estradiol group (0.83±0.32 vs. 0.17±0.20; p=0.102).

No significant differences were observed in percent ovulation (78.5% vs. 84.9%, p=0.289), the number of CLs on day 5 post artificial insemination (AI) (12.2±1.36 vs. 10.3±0.63, p=0.280), embryo recovery rate (47.95% vs. 45.16%), and total number of recovered embryos (5.83±0.86 vs. 4.67±1.16, p=0.328) in the estradiol group compared to the conventional group, respectively. Further, a mean number of transferable embryos recovered were non-significantly higher in the estradiol group compared to the conventional group (3.67±0.93 vs. 2.67±0.68, p=0.437).

The administration of LH or GnRH did not have any significant effect on ovulation rate and a number of embryos recovered in buffaloes (Table-2). However, the proportion of transferable embryos collected from buffaloes treated with LH were significantly higher in LH group compared to GnRH group (73.3% vs. 48.5%, p=0.044) having lower mean number of anovulatory follicles (1.±0.63 vs. 2.0±1.62) on the day of flushing.

Discussion

In vivo embryo production could be a viable option in buffaloes on the identification of good donors. However, the detection of estrus is more difficult in buffaloes than cattle which made implementation of the conventional superstimulatory protocols more difficult in buffaloes resulting in poor success rates. The initiation of superstimulatory treatment following exogenous control of follicular wave emergence using estradiol-17β could eliminate the need for estrus detection and avoid the unnecessary waiting period until mid-cycle to initiate the conventional protocol. Therefore, the present work was undertaken to study the follicular dynamics, superovulatory response, embryo recovery following superstimulation protocol initiated at the beginning of estradiol-17β-induced follicular wave emergence and its comparison with the conventional protocol in buffaloes.

Follicular dynamics

The number of small follicles (≤8 mm) present at the time of initiation of superstimulatory treatment was significantly higher in the estradiol group compared to the conventional group (Table-1). Similarly, following the treatment, a higher number of follicles reached ovulatory size (≥8 mm) in the estradiol group (p=0.054) compared to the conventional group on the day of estrus. The average number of follicles reaching ovulatory size was over 12 and 15 in the conventional and the estradiol groups, respectively. Our results corroborated the findings of Baruselli et al. [13] and Carvalho et al. [14] who showed that
a sufficient number of follicles reach ovulatory size.
A higher number of follicles reaching an ovulatory
size in superstimulated buffaloes using FSH alone or
FSH + pregnant mare’s serum gonadotropin had been
reported by Abd-Allah et al. [15]. However, the ear-
erlier studies had used mainly conventional protocols
for superstimulation and obtained a poor response in
buffaloes. These studies pointed out the low popula-
tion of ovarian primordial follicles in buffaloes com-
pared to cattle as the possible cause for low superovul-
tory response in buffaloes [4,16]. However, Azawi
et al. [17] and Campanile et al. [18] reported the
recruitment of a similar number of ovarian follicles in
buffaloes and cattle during wave emergence.

The reason for higher number of follicles reaching
ovulatory size (≥8 mm) in the estradiol group com-
pared to the conventional group in the present study
could be emergence of a new follicular wave 4 days
later following administration of estradiol-17β [10]
which led to availability of significantly higher num-
ber of smaller follicles of uniform size to undergo
superstimulation. The lower number of the small fol-
cicle at the initiation of superstimulatory treatment
could be due to asynchrony of the emergence of sec-
ond follicular wave and initiation of superstimula-
tory treatment during mid-cycle in the conventional
group compared to the estradiol group. In addition to
that the presence of dominant follicles of ≥8 mm size
(p=0.102) in three buffaloes at the beginning of super-
stimulatory treatment might have exerted an inhibi-
tory effect on the growth of subordinate follicles in the
conventional group.

Ovulation rate

In general, the both groups showed higher ovula-
tion rate (74% and 84%) in the present study. The sim-
ilar high ovulation rate in superstimulated buffaloes
has been reported by Lipinski et al. [19]. The moderate
ovulation rate (~60%) and number of CLs (~9 CL) in
buffaloes have been reported by Baruselli et al. [13].
However, the superstimulation studies carried out in
90s, the ovulation rate was much lower and ranged
between 2 and 4 ovulations in buffaloes [20,21]. Patel
et al. [22] also reported fewer ovulations (~4) follow-
ing superstimulation in pandharpuri buffaloes.

The superovulatory response in terms of num-
ber of CLs on the 5th day post AI in both groups was
higher than reported by Misra and Joshi [20] and
Agarwal et al. [21]. Misra [6] in a study reported that
12% of the superstimulated buffaloes developed
>10 CLs. The higher number of CLs in both groups
in this study could be due to the better synchrony of
beginning of superstimulatory treatment and wave
emergence in the estradiol group. In the conventional
group, it could be due to better detection and induction
of estrus (day=0) than previous studies which did not
use ultrasonography to establish a day of estrus. The
use of 5-day protocol over the 4 days superstimula-
tory protocol and administration of LH or GnRH for
facilitating the process of ovulation could be another
reason for higher ovulation and the number of CLs in
the present study. It was observed that use of 5-day
FSH protocol gave better results compared to 4 days
dosage regimen.

The comparatively higher number of CLs in the
estradiol group than the conventional group on the
5th day post AI could be due to the higher number of
follicles reaching ovulatory size (≥8 mm) in the estra-
diol group compared to the conventional group. The
presence of higher number of dominant follicles at the
initiation of superstimulatory protocol in the conven-
tional group could be another reason. The decreased
superovulatory response due to the presence of dom-
inant follicle has also been reported by others [23].
Li et al. [24] reported that the immunoneutralization
against inhibin or follistatin was effective in enhanc-
ing ovarian follicular development, ovulation rate,
and a number of embryos recovered from superstim-
ulated buffaloes.

Embryo recovery

The moderate embryo recovery rate of ~45-48
was observed in the present study. The recovery
rate achieved in the present study was higher than
(~34%) reported by Baruselli et al. [13] and Carvalho
et al. [14] but lower than 63-80% reported in cattle.
Misra [6] also reported recovery rate of >60% and
observed that recovery decreased to 33% when a
number of ovulations were more than 15 per buffalo
donor. It was proposed that lower embryo recovery in
buffaloes could be due to changed uterine environ-
ment as a result of high estrogen level [25], failure
of oocyte capture and/or of oocyte transport along the
oviduct [13], fragile connection between the oocyte
and granulosa cells [26] higher number of anovula-
tory follicles, a more rigid ovary-mesovarium con-
nection, and a thicker infundibulum muscle layer than
cattle [27,28], however none of these aspects has been
critically studied. The effort to improve embryo recov-
ery by giving progesterone during the periovulatory
period [29] was unsuccessful due to the overall failure
of ovulation. The use of recombinant bovine somato-
tropin to improve fragile connections between oocyte
and granulosa cells also proved inconclusive [14]. The
exact cause of this low efficiency in embryo recovery
is still unknown.

The average number of embryos recovered in the
present study was higher in the estradiol group than
the conventional group. The two studies in the liter-
ature have reported the recovery of similar or higher
number of embryos. Misra et al. [30] have recovered
up to mean of 7.5 embryos from 10 superstimulated
buffaloes in India and Jiang et al. [31] reported the
recovery of average six embryos per superovulation
in China. In another study, Wang et al. [32] reported
the higher recovery of eight embryos per animal after
sacrifice with a recovery rate of 56.5%, out of which
seven were of transferable grade. Few other studies
involving immunoneutralization against inhibin or follistatin [24] and administration of PGF2α during the periovulatory period [33] has also found effective in enhancing a number of embryos recovered from superstimulated buffaloes.

**Transferable embryos**

The average number of transferable embryos recorded in the estradiol group was non-significantly higher than the conventional group. Misra et al. [34] reported an average recovery of 4.2 total embryos and 2.1 viable embryos per flushing using purified FSH-P1 preparation. In isolated trials transferable embryos as high as 5.9 [30], >4 transferable embryos [31] and 7 transferable embryos after sacrificing [32] has also been achieved. The average number of transferable embryos in our study was higher than many other studies reporting only one to two transferable embryos in buffaloes [4,16,20,21]. The percentage transferable embryos achieved in both groups were comparable to Carvalho et al. [14], Heleil and El-Deeb [23] and lower than reported by Soares et al. [33] achieved using protocol involving administration of PGF2α during periovulatory period.

**Effect of LH and GnRH on superovulatory response**

Differences in superovulatory response, embryo recovery and a number of transferable embryos were non-significant between buffaloes administered LH or GnRH in the present study (Table-2). However, the percentage of transferable embryos was significantly higher in buffaloes treated with LH (p=0.044) compared to GnRH group. The high estradiol level due to the presence of higher mean value of anovulatory follicles (2.0 vs. 1.0) on the day of embryo collection might be responsible for a lower percentage of transferable embryos in buffaloes administered with GnRH. Beg et al. [8] reported that buffaloes were more sensitive to higher estradiol levels than bovine which might have affected their uterine environment during superstimulation [25].

Techakumphu et al. [35] reported that supplementation of GnRH at 8-12 h after standing heat seemed to produce more transferable embryos than those treated at standing heat or the controls. Zicarelli et al. [36] did not find any significant differences in ovarian follicular response in buffaloes treated with GnRH agonist LH protocol (involving administration of LH as an ovulation inducer) compared to the conventional protocol. However, Carvalho et al. [10] reported that the GnRH agonist LH protocol was effective in improving ovulation rate in superstimulated buffaloes. Qin et al. [37] observed higher ovulation rate in buffaloes administered luteinizing hormone releasing hormone-A3 (GnRH) compared to LH.

**Conclusion**

Significantly higher number of growing follicles was present in the estradiol group at the initiation of superstimulatory treatment compared to the conventional group. Superovulatory response in terms of number of CLs, total, and transferable embryos tend to be higher in the estradiol group compared to the conventional protocol indicating that the estradiol based protocol could be successfully used for superstimulation in buffaloes. In addition to this, the administration of LH in buffaloes subjected to fix timed AI following superstimulation produced a significantly higher percentage of transferable embryos compared to administration of GnRH. Therefore, the results of the present study indicated that initiation of superstimulatory treatment subsequent to the synchronization of follicular wave emergence by estradiol-17β and fixed timed AI could be successfully used for embryo production in buffaloes.

**Authors’ Contributions**

The present study was part of NS’s PhD dissertation. The work was designed by GSD and PSB. The execution of experimentation protocol and performing ultrasound scanning was done by NS, VSM, and MH. The lab work was performed by NS, SS, and DD. Statistical analysis and drafting of the manuscript were done by NS, GSD, and DD. All authors read and approved the final manuscript.

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**Competing Interests**

The authors declare that they have no competing interests.

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