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*Research Article*

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# Ultrasonographic monitoring of ovarian activities after superovulation treatment in buffalo cows

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**Abstract**

Potential applications of modern reproductive technologies including monitoring follicular dynamics, estrus synchronization and superovulation would be a new dimension of research at the farm level for improving buffalo's fertility and production. Therefore, the study was aimed to understand and monitor the ovarian dynamics after induction of superovulation in buffalo cows. Ovarian activities after superovulation treatment were monitored by trans-rectal ultrasonography. Follicle stimulating hormone (FSH) was used for superovulation in six mature Murrah buffalo cows and the embryos were collected by non-surgically technique on day 6 of the estrous cycle. The result showed a significant ($P < 0.05$) effect of superovulation treatment on follicular size, number and maturation. The result also revealed that the number and size of follicles of right and left ovaries, as well as their growth rate, have no significant ($P > 0.05$) difference during the superovulation treatment of animals. The development of dominant follicle and formation of corpus luteum after treating with the FSH had shown positive results in the present study but the number of recovery of embryos was very poor. Observed the ovulation rate was 58.62% and the embryo recovery rate was very poor ($\sim$ 3%). This study suggests that more efforts should be made to better comprehend the mechanism of embryo production, maturation and collection during the MOET program in buffalo cows.

**Keywords:** Ovarian activities, superovulation, ultrasonography, buffalo.

## INTRODUCTION

Management and appropriate rearing procedures including reproductive biotechnologies which are unquestionably the most applied techniques in the field of life sciences and livestock farming. these new technologies have significantly contributed to planning selective breeding as well as distribution of elite buffalo genes, the reduction in generation interval and continued genetic gain resulting ton produce production of buffalo meat and milk (Thibier, 2005). Multiple ovulation and embryo transfer (MOET) programs which use in dairy cattle breeding since the commercialization of the industry in the early 1970s, enhancing the production of multiple offspring from genetically superior females by reducing generation intervals and this biotechnology has been made in boosting milk production, improving reproduction, more rapid growth of the animals and controlling the diseases. The efficiency of a superovulation procedure is evaluated in terms of the numbers of viable embryos, pregnancies and live calves. Success-

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ful embryo transfer in buffaloes was first reported by Drost *et al.* (1983) followed by Vlakhov *et al.* (1985) in Bulgaria and Misra *et al.* (1988) and Madan *et al.* (1989) in India. Although it has been done more than three decades ago, the application of these techniques in buffaloes is still limited due to the poor efficiency compared to cattle after superovulation (Carvalho *et al.*, 2002; Drost, 2007). The poor responses are due to inherent endocrine diversity, the characteristics of follicular population and folliculogenesis, hormonal profile during the superovulation cycle and failure to recovery embryos due to different embryos transport speeds or due to non-synchrony of uterine or ovarian events. However, the application of MOET in buffalo still needs to be improved. Therefore, this was conducted to monitor ovarian activities after superovulation treatment of buffalo cows by transrectal Ultrasonography.

# MATERIALS AND METHODS

## Selection of donor buffaloes and management

The study has been conducted at the Central Institute for Research on buffaloes (CIRB), Hisar, Haryana, India, using six mature Murrah buffalo cows with a live weight of 400 kg to 600 kg. Donor buffalo cows met all criteria of donor selection like superior traits, calved at least one, normal estrus cycle, service per conception $< 2$, good health and disease-free were housed and maintained properly. Each buffalo was offered concentrate feed supplementation as a standard feeding schedule. Drinking water and green grass were offered *ad libitum*.

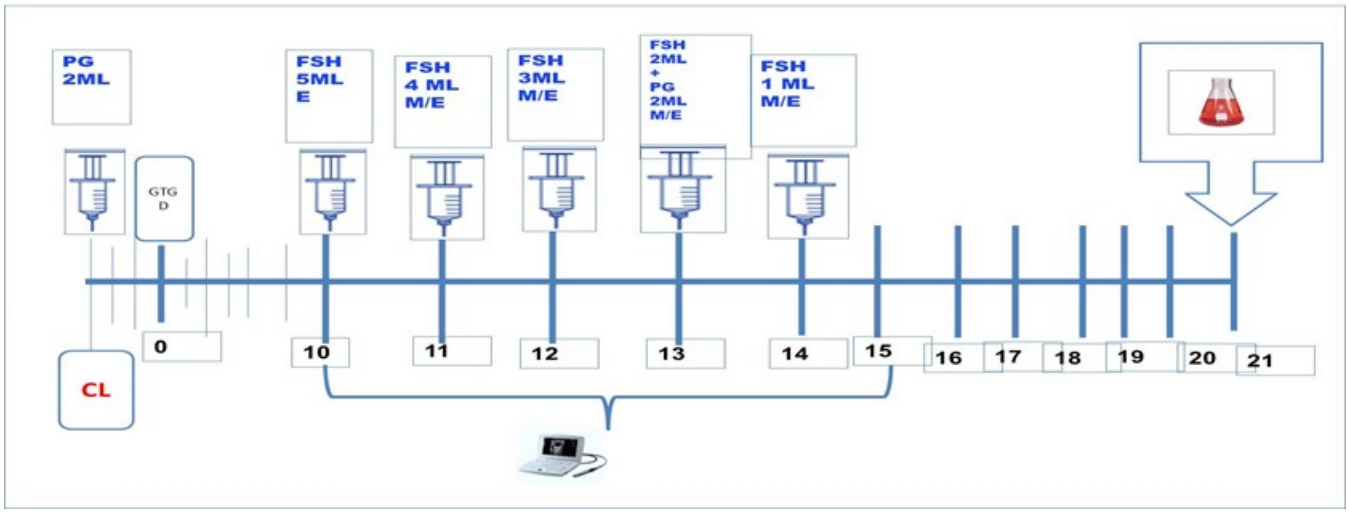


Figure 1: Superovulation protocol CL= Corpus luteum, FSH=Follicle Stimulating Hormone, M=Morning, E=Evening, PG=Prostaglandin

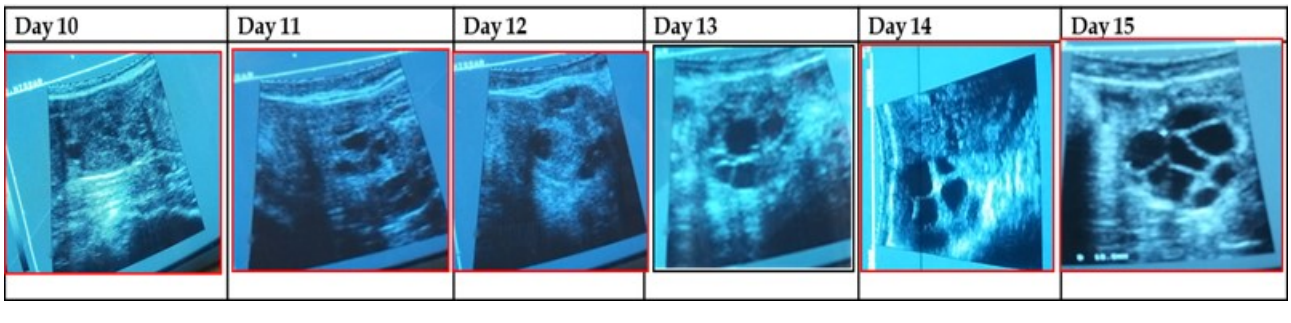


Figure 2: Follicles and their growth pattern observed by transrectal ultrasonography during superovulation treatment.

### Superovulation and AI in donor buffaloes

In presence of CL observed by transrectal ultrasonography, all donor buffaloes were given one intramuscular injection of 2 ml prostaglandin (Pragma-Bharti Life Science, Pelhar Vasai Fata, Vasai East District-Thane-401208) to induce estrus. The day when animals showed estrus, was considered Day 0. On day of 10 of estrus cycle, 5 ml of FSH (Each ml contains 20 mg NIH-FSH-P1) (FOLLTROPIN®-V, Bioniche Animal Health Canada) were administrated to each buffalo twice daily (12 hours interval) intramuscularly in decreasing manner (5.0; 5.0; 4.0; 4.0; 3.0; 3.0; 2.0; 2.0 and 1.0; 1.0 ml) for 5 days. On the morning of Day 13 (after the 6th injection of Folltropin), 2 ml prostaglandin was injected intramuscular and was repeated after 12 hours. On Day 15 morning, buffaloes showing estrus were artificially inseminated with frozen semen, repeated in the evening and Day 16 morning of the estrous cycle. Superovulation treatment is shown in Figure 1.

### Monitoring of donor buffalo cows

Ultrasonography was carried out daily to assess the follicular dynamics using an ultrasound machine equipped with a B-mode micro convex intraoperative probe using 7 MHz from the beginning to the end of treatment. Further ultrasound examinations were carried out on the day of flushing to check the ovulation rate as well as luteal dynamics. In every ultrasound examination, total numbers of small ($<$ 3mm), medium ($\geq$ 3 to 8mm) and large ($\geq$ 10mm) ovarian follicles were recorded. Figure 2 shows the growth pattern of follicles during superovulation treatment with FSH injection.

### Embryo collection

The embryos were collected on Day 21 using the non-surgical technique. Buffalo was confined in a Travis and 2ml of 2% Xylocaine Hydrochloride was injected as low epidural anaesthesia to prevent straining and defecation. Transrectal ultrasound examination was made for observing the ovary and number of corpora lutea (CL). A 2-way Woerleine catheter was used to collect the embryos. The Woerleine catheter was inserted through the vagina, cervix and into one of the uterine horns where the balloon was inflated. Usually, 8 to 10 cc of air were used in buffalo species. The inflated balloon was sealed off the anterior portion of the uterine horn, avoiding fluid from getting out of the uterus when the flushing medium is inserted into the uterine horn to recover the embryos. Each horn of the uterus was flushed with 400 ml Dulbecco's Phosphate Buffered Saline (DBPS) containing 0.1% Bovine Serum Albumin (BSA). When the flushing was completed, the cuff was deflated and the contents of the catheter were carefully allowed to flow into a sterile holder located at the end of the outflow tubing. Immediately after flushing, the collected flush media was observed using a stereo microscope for embryos. Embryos collected was transferred to fresh DPBS containing 0.4% BSA for evaluation morphologically and were graded as per the manual of the International Embryo Transfer Society (Robertson and Nelson, 1998).

### Statistical analysis

The average number and size of follicles of right and left ovary; the average growth rate of follicles during superovulation program; average size and number of follicles at flushing time; as well as total number and size of DF and CL formation of both ovaries were analyzed by using ANOVA test. Data were presented as mean±standard error. Differences were considered statistically significant when they reached at least the 5% confidence level. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software version 20.

Table 1: Total number, size and growth rate of follicles between right and left ovary of donor Murrah buffalo cows during superovulation treatment.

| Murrah cows | Sonographic observation | Right ovary | Left ovary |
|---|---|---|---|
| N=6 | Total number of follicles | $6.88 \pm 0.38^{NS}$ | $7.54 \pm 0.55$ |
| | Size of follicles (mm) | $7.98 \pm 1.01^{NS}$ | $8.35 \pm 0.91$ |
| | Follicular growth rate (mm/day) | $1.21 \pm 0.14^{NS}$ | $1.01 \pm 0.52$ |

Values are Mean±SE. $^{NS}$ Non-significant at $P > 0.05$ within the row.

## RESULTS

The total number, size and growth rate of follicles between the right and left ovary of donor Murrah buffalo cows during superovulation treatment is presented in Table 1. The total number, size and growth

rate of follicles in right vs. left ovary were $6.88 \pm 0.38$ vs. $7.54 \pm 0.55$, $7.98 \pm 1.01$ vs. $8.35 \pm 0.91$ mm and $1.21 \pm 0.14$ vs. $1.01 \pm 0.52$ mm/day, respectively in donor Murrah buffalo cows during superovulation treatment. The total number and size of follicles were non-significantly ($P > 0.05$) lower in the right ovary than the left ovary, but the follicular growth rate was non-significantly ($P > 0.05$) higher in the right ovary than left ovary of donor Murrah buffalo cows during superovulation treatment.

Table 2: Number and size of follicles in donor Murrah buffalo cows (n=6) during superovulation treatment

| **Follicular groups** | **Parameters** | **Day during superovulation treatment** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 10 | 11 | 12 | 13 | 14 | 15 |
| SFL ($\leq$ 3mm) | No. of follicles | $4.66 \pm 0.56^{a}$ | $4.00 \pm 0.85^{a}$ | $1.67 \pm 0.49^{b}$ | $1.17 \pm 0.30^{b}$ | $1.83 \pm 0.47^{b}$ | $1.00 \pm 0.44^{b}$ |
| MFL ($> 3-8$ mm) | No. of follicles | $5.66 \pm 1.20^{bc}$ | $9.83 \pm 1.72^{a}$ | $9.50 \pm 1.28^{a}$ | $8.16 \pm 0.74^{ab}$ | $3.66 \pm 1.02^{c}$ | $2.66 \pm 06^{c}$ |
| | Diameter of follicless | $5.56 \pm 0.34^{b}$ | $5.64 \pm 0.15^{b}$ | $6.27 \pm 0.21^{a}$ | $6.78 \pm 0.18^{a}$ | $6.94 \pm 0.23^{a}$ | $5.84 \pm 0.48^{b}$ |
| LFL ($> 8$ mm) | No. of follicles | $0.17 \pm 0.16^{c}$ | $0.83 \pm 0.30^{c}$ | $3.33 \pm 1.02^{b}$ | $6.33 \pm 1.25^{b}$ | $11.0 \pm 1.9^{8}$a | $12.0 \pm 1.34^{a}$ |
| | Diameter of follicless | $9.00 \pm 0.00^{c}$ | $9.15 \pm 0.13^{b}$ | $9.70 \pm 0.29^{b}$ | $10.51 \pm 0.21^{b}$ | $11.48 \pm 0.27^{a}$ | $12.33 \pm 0.12^{a}$ |

Values are Mean±SE. [a,b,c] Values differed significantly at $P > 0.05$ within the row

Table 3: Number and size of follicles in superovulated donors Murrah buffalo cows (N=5) on the day of flushing

| **Parameters** | **Follicles** | | |
|---|---|---|---|
| | SFL ($\leq$ 3mm) | MFL ($> 3-8$mm) | LFL ($> 8$mm) |
| Number of follicles | $3.66 \pm 0.42^{a}$ | $1.50 \pm 0.56^{b}$ | $3.50 \pm 0.62^{a}$ |
| Diameter of follicles | $3.00 \pm 0.00^{c}$ | $5.50 \pm 0.43^{b}$ | $9.45 \pm 1.30^{a}$ |

Values are Mean±SE. [a,b,c] Values differed significantly at $P > 0.05$ within the row.

The number and size of follicles in donor Murrah buffalo cows during superovulation treatment is presented in Table 2. The number of small follicles ($\leq$ 3mm) was significantly ($P < 0.05$) lower at day 15 than day 10–11, and it was non-significantly ($P > 0.05$) differed at day 12–15. Simultaneously, the number of medium follicles ($> 3-8$mm) was significantly ($P < 0.05$) lower at day 15 than day 11–13 and it was non-significantly ($P > 0.05$) differed at day 10, 14 and 15. The number of large follicles ($> 8$mm) was significantly ($P < 0.05$) higher observed on day 14–15 than on day 10–13. The diameter of medium follicles ($> 3-8$mm) was significantly ($P < 0.05$) lower at day 15 than day 12–14, and it was non-significantly ($P > 0.05$) differed at day 10, 11 and 15. Whereas, the diameter of large follicles ($> 8$mm) was significantly ($P < 0.05$) higher at day 14–15 than day 10–13, and it was non-significantly ($P > 0.05$) differed at day 10–13.

Table 3 shows the number and size of follicles in superovulated donors Murrah buffalo cows on the day of flushing. The number of medium follicles ($> 3-8$ mm) was significantly ($P < 0.05$) lower compared to the small ($\leq$ 3mm) and large follicles ($> 8$mm). The diameter of large follicles was significantly ($P < 0.05$) higher in comparison to small and medium follicles.

Effect of FSH on different variables during superovulation treatment and flushing output in superovulated donors Murrah buffalo cows were observed and results are shown in Table 4. Six Murrah buffalo cows were

treated by FSH for superovulation. Out of six there are five cows were responded and a total of 58 numbers of dominant follicles were produced with $11.6 \pm 1.16$ ovulatory follicles per cow. There are about 34 numbers of ovulation and CL formation was observed in the responded cows with $6.8 \pm 0.96$ per cow, where anovulatory follicles were $4.8 \pm 0.86$ per cow. The ovulation rate was 58.62%. It is observed that only one embryo was recovery after flushing with $0.20 \pm 0.20$ (Mean±SE) per cow and the embryo recovery rate was 2.94%. The only one recovered embryo after flushing in this experiment was viable.

Table 4: Effect of FSH on different variables during superovulation treatment and flushing output in superovulated donors Murrah buffalo cows.

| **Variables** | **Quantity** | **Mean±SE** |
|---|---|---|
| Number of Murrah buffalo cows | 6 | - |
| Responded Murrah buffalo cows | 5 | - |
| Number of dominant follicles | 58 | - |
| Ovulatory follicles | - | $11.6 \pm 1.16$ |
| Number of ovulation and CL formation | 34 | $6.8 \pm 0.96$ |
| Anovulatory follicles | - | $4.8 \pm 0.86$ |
| Ovulation rate (%) | 58.62% | - |
| Recovery embryo after flushing | 1 | $0.20 \pm 0.20$ |
| Embryo recovery rate (%) | 2.94% | - |
| Viable embryo | 1 | $0.20 \pm 0.20$ |

# DISCUSSION

Superovulation requires an increased number of preovulatory follicles by the administration of gonadotrophins stimulating the effect of FSH. Though multiple ovulation and embryo transfer are extensively practiced in bovine, 60 to 70% of recovered embryos are suitable for transfer in cattle, Superovulatory response is much lower in buffalo. Therefore, the use of this technology in the buffalo is much more limited. The study was performed to monitor the ovarian structures using ultrasonography after superovulation treatment in Murrah buffalo cows.

The result illustrated that the number of small follicles ($\leq 3$ mm) present at the time of initiation of super stimulatory treatment is significantly varied compared to the day 15 or end of the treatment. Similarly, following the treatment, a higher number of follicles reached ovulatory size ($\geq 8$ mm) compared to the day of treatment of the donor's animals. The average number and size of follicles reaching ovulatory size are $12.00 \pm 1.34$ and $12.33 \pm 0.12$ mm respectively and these results have supported the findings of Baruselli *et al.* (2000) and Carvalho *et al.* (2007) who showed that a sufficient number of follicles reach ovulatory size after FSH treatment. A similar finding was also reported in buffalo cows when super stimulation of ovulation was performed by using FSH alone or FSH +with pregnant mare's serum gonadotropin (PMSG) (Abd-Allah *et al.*, 2013). The initiation of super stimulatory treatment during the mid-cycle of the estrus of cyclic buffalo cows could have a positive effect on growing follicles through initiating new follicular waves resulting in increased numbers of ovulatory follicles as stated by Carvalho *et al.* (2007).

In our study, the number of large size of follicles at day 10 was very few, only $(0.17 \pm 0.16)$ number but at day 15 numbers were more $(12.00 \pm 1.34)$ might be due to the effect of FSH during the treatment of superovulation. Generally, the follicles are growing gradually day by day with the effect of FSH, which is a glycoprotein hormone that plays a major role in the development and maturation of ovarian follicles before the release of an ovum from one follicle at ovulation, increases estradiol production and the secretion of gonadal hormones (Kim *et al.*, 2013).

The result illustrated that the average numbers of small $(3.66 \pm 0.42)$ and large $(3.50 \pm 0.62)$ sized follicles were significantly ($P < 0.05$) higher than the number $(1.50 \pm 0.56)$ of medium-sized follicles. Similarly, there was significant ($P < 0.05$) differences in the average diameter of the small $(3.00 \pm 0.00$ mm), medium $(5.50 \pm 0.43$mm) and large follicles $(9.45 \pm 1.30$ mm). The result revealed that the ovulation rate was 58.62% and this moderate ovulation rate is slightly lower than the finding of Baruselli *et al.* (2000) who reported that ovulation rate ($\sim 60\%$) in buffalo cows. In contrast, Patel *et al.* (2010) reported fewer ovulations ($\sim 4$) following super stimulation in pandharpuri buffalo cows. This study also reported the presence of a greater number of dominant follicles at the time of flushing the uterus. It may be one of the causes of the presence of high plasma concentration levels of estrogen, which altered the estradiol: progesterone ratio and as a result, the embryo recovery rate was very poor, as found by Beg and Ginther

(2006). The embryo recovery rate of $\sim$ 3% was very poor in the present study. The recovery rate achieved in the present study is lower than that reported by Baruselli *et al.* (2000) ($\sim$ 34% in buffaloes) and (63-80% in cattle). Observation of a higher number Carvalho *et al.* (2007) of large follicles on the day of estrus and the number of CL on the day of flushing using trans-rectal ultrasonography indicates good ovarian and hormonal responses to superovulation treatment in buffalo. However, a poor number of the viable embryos might be due to several reasons such as difficulty in the ovum capture by the oviduct fimbria resulting from the turgidity of the genital system because of high estrogen level (Misra *et al.*, 1998), failure of oocyte capture and/or of oocyte transport along the oviduct (Baruselli *et al.*, 2000), the more fragile connection between the oocyte and granulosa cells (Gasparrini, 2002), the inability of fimbria to trap ova from enlarged superovulation ovary, a higher number of anovulatory follicles, a more rigid ovary-mesovarium connection and presence of a thicker infundibulum muscle layer (Carvalho *et al.*, 2011, 2012) etc. Several reports on unsuccessful attempts to improve recovery rate by using recombinant bovine somatotropin to improve fragile connections between oocyte and granulosa (Carvalho *et al.*, 2007) and by giving progesterone during the periovulatory period (Soares *et al.*, 2013) indicates to further studies to find out the exact cause of this low efficiency in embryo recovery in buffaloes. Estradiol plasma concentrations are particularly high during superovulation (Beg and Ginther, 2006), as a result, the altered estradiol: progesterone ratio, which may impair the interaction between the ovum and the ciliated cells of endosalpinge during the ovulation which may influence oocyte recruitment by the fimbriae. In a study, Carvalho (2006) found that buffalo's ovary adheres more firmly to the mesovarium than the cow's ovary, which may act as an important cause for the failure of fimbriae to collect the ovulated ovum. The main objective of the superovulation treatment is to obtain a high number of embryos from high genetic merit subjects (Mapletoft *et al.*, 2002; Mapletoft and Bó, 2012). But, in comparison to cattle, MOET with a relatively low embryo recovery rate as well as unfertilized ova is reported by a lot of researchers (Misra and Tyagi, 2007; Neglia *et al.*, 2010; Qin *et al.*, 2012) around the world. Our study result is more or less similar to these findings. This study concludes that the response of buffalo to superovulation protocol allows a good Superovulatory response, both in terms of the number and size of follicles and in terms of ovulation, without improving the embryo recovery rate. Therefore, this suggests more efforts to better comprehend the mechanism and factors involved in the failure of ovum capture by the oviduct to improve the MOET in buffaloes.

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## CONFLICT OF INTEREST

The authors declare no potential conflict of interest concerning this research.

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