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Effect of 2 Day Versus 3 Day Superstimulation Protocol on *In Vivo* Maturation of OPU Derived Oocytes in Buffaloes

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ABSTRACT

The study was planned to evaluate the effect of two superstimulation protocols (2-Day vs. 3-Day) on *in vivo* maturation of oocytes in buffaloes. Buffaloes (n=8) were superstimulated twice using 2x2 design at an interval of 30-35 days using 200 mg and 300 mg FSH divided into four and six doses (each dose of 50 mg) given over 2 and 3 days at 12 hourly intervals, respectively. Prostaglandin $F_{2\alpha}$ analogue administered (500 µg Cloprostenol; i/m) at 72 and 84 hrs after start of superstimulatory treatment. Oocytes were retrieved from >6 mm follicles size at 48h after the last FSH injection by ovum pick up. Oocyte maturation was assessed by the degree of cumulus cell expansion and by Orcein staining of denuded oocytes. OPU of 60 and 89 follicles resulted in recovery of 38 and 50 cumulus oocyte complexes (COCs) in 2-Day and 3-Day group, respectively. Significantly higher proportion of expanded oocytes was recovered in 3-Day protocol compared to 2-Day protocol (36.0% *vs.* 13.2%) and higher number of compact COCs were recovered in 2-Day protocol. In 3-Day protocol, significantly higher percentage of oocytes retrieved was at Metaphase-II stage compared to 2-Day protocol (40% vs. 15.8%, respectively). The preliminary trial demonstrated that 3-day superstimulatory protocol yielded higher percentage of *in vivo* matured oocytes than 2-Day protocol in buffaloes.

Key words: Buffalo, Oocyte, Ovum pick-up, Superstimulation.

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INTRODUCTION

Buffalo is an economically important dairy animal in India and many other Asian countries. Buffalo is considered as a difficult breeder due to its inherent susceptibility to environmental stress, delayed onset of puberty, poor overt signs of estrus and larger inter-calving period. Buffalo contributes nearly half of the country's total milk output

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(198.4 million tonnes in 2019-20) to the national pool. However, the average milk yield per lactation of buffaloes is still very low and there is need to implement assisted reproductive techniques in order to increase the number of high milk producing buffalo.

Multiple ovulation embryo transfer (MOET) program has played an important role in faster dissemination of superior germplasm in cattle. However, the application of this technology has not yielded desired results in buffaloes due to low recovery of transferable embryos (Drost, 2007, Singh *et al*., 2016). Currently, use of *in vitro* production of embryos (IVP) is increasing, as it is considered superior than conventional embryo transfer program in producing more number of transferable embryos per unit of time (Ferre *et al*., 2019). There is need to harvest the benefits associated with this technology to improve buffalo germplasm. However, production of fewer transferable embryos due to low recovery of immature oocytes (1 to 2 per buffalo) and failure of large number of oocytes to develop into transferable embryos are the two major limiting factors of IVP in buffaloes (Nandi *et al.,* 2002, Mishra *et al.,* 2008).

Successful maturation of oocytes is considered the first and most critical step of IVP. The studies in cattle have shown that *in vivo* matured oocytes have higher developmental competence than *in vitro* matured oocytes (Yadav *et al.,* 2006). It has been observed that superstimulation with FSH and *in vivo* maturation has improved recovery and developmental competence of oocytes in cattle (Blondin *et al.,* 2002). However, the similar studies are lacking in buffaloes. Therefore, establishment of suitable superstimulation and *in vivo* maturation protocols could help in enhancing number of transferable embryos by IVP in buffaloes. In view of this, a preliminary trial was planned to evaluate the effect of 2 Day *vs.* 3 Day superstimulation protocol on *in vivo* maturation of oocytes in buffaloes. As per the knowledge of authors, this was the first trial of its kind undertaken to evaluate *in vivo* maturation of oocytes in buffaloes.

MATERIALS AND METHODS

Superstimulatory treatment groups: Normal cyclic, healthy pluriparous buffaloes (n=8) were injected 2 mg of Estradiol-17β along with intravaginal placement of 1.38 mg progesterone implant (Eazibreed CIDR) at random stage of estrous cycle (day 0) followed by initiation of superstimulation treatment on day 4 onward. Buffaloes divided in two groups were superstimulated twice (Fig. 1) in 2 x 2 reciprocal design at an interval of 30 to 35 days between successive treatments.

Group I: 2 Days protocol - Buffaloes (n=8) were superstimulated using 200 mg follicle stimulating hormone (FSH) administered in four equal doses at 12 hour intervals.

Group II: 3 Days protocol - Buffaloes (n=8) were superstimulated using 300 mg of FSH given in six equal doses at 12 hour intervals.

All the buffaloes were injected prostaglandin $F_{2\alpha}$ analogue (500 µg Cloprostenol; i/m) at 72 and 84 hrs after start of superstimulatory treatment and CIDR was removed at the time of second prostaglandin injection. Half of the buffaloes (n=4) in each group were administered luteinising hormone (LH) (Lutropin-V: Bioniche Animal Health) 25 mg about 6 hour before OPU and the other half did not receive LH.

Ovum pick up: The follicles measuring >6 mm of size were subjected to OPU after 48 hours of last FSH injection in both the groups. Ovum pick up was performed using ultrasound scanner (Aloka SSD-500, Tokyo, Japan) equipped with a 5-MHz transvaginal transducer using 80 mm Hg vacuum pressure (Singh *et al.,* 2016). Before OPU, buffaloes were administered 0.02 mg/kg Xylazine HCl for tranquilization and 3-5 ml Xylocaine 2% for epidural anaesthesia. The oocytes collection medium was Dulbecco's phosphate buffer saline supplemented with 0.3% BSA, 2 IU/ml heparin and 50 µg/ml Gentamicin. The number and size of follicles on each ovary were determined before puncture. Visible follicles measuring > 6mm diameter were aspirated and follicular fluid collected in 50 ml tube (1 tube per buffalo) was kept in thermostatically controlled dry bath.

Oocyte maturation assessment: The aspirated follicular contents of each animal were filtered through 75µ Em-Con filter (Bioniche, Canada) and rinsed with plain dPBS. The contents of the filter (10-15 ml) were poured into 90 mm petridish (Falcon) for oocyte searching under a stereomicroscope (model-SZX7, Olympus, Japan). The maturation of oocytes was assessed by the degree of expansion of cumulus cell mass as well as by orcein staining after denudation of oocytes.

Cumulus cell expansion: Based on the degree of cumulus cell expansion, the oocytes were assessed after maturation and classified according to Hunter and Moor (1987) as: full cumulus cell expansion (Expanded) - enlargement of the cumulus cell mass at least 3x diameters away from the zona pellucida; moderate cumulus cell expansion (Intermediate) - expansion in order of 2x diameter away from the zona pellucida; no expansion (Compact) - no evident change or slight expansion of cumulus cells.

Nuclear maturation by orcein staining: Oocytes were stained and evaluated as described by Hunter and Polge

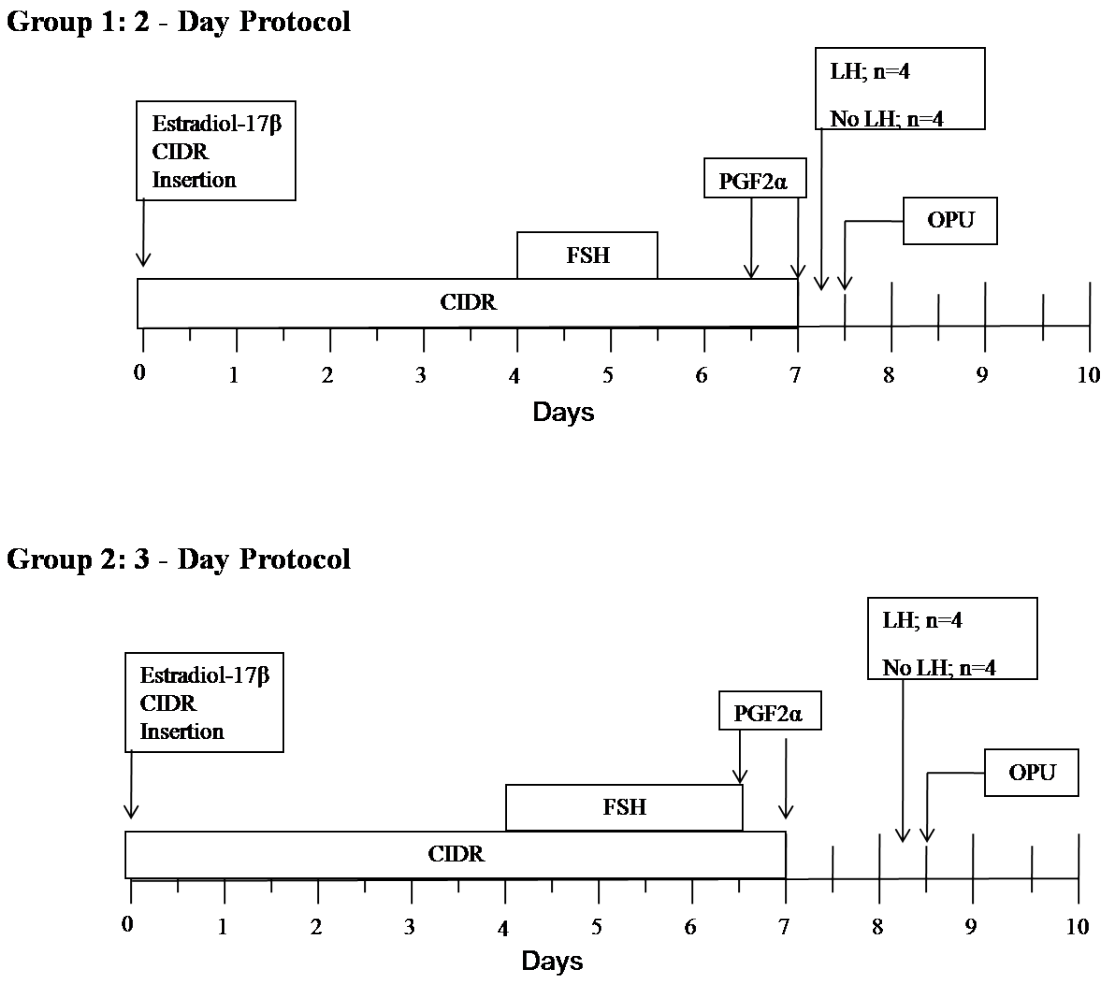

Fig. 1: Schematic diagram of 2-Day and 3-Day protocol used for superstimulation of buffaloes in group 1 and group 2, respectively.

(1966). Briefly, oocytes were denuded using 0.1% (w/v) hyaluronidase in calcium & magnesium free dPBS. Five to ten denuded oocytes were mounted on glass slide under coverslip (supported with paraffin-vaseline corners) and fixed in ethanol : acetic acid (3:1, v/v) for 24 hours. Then, oocytes were stained in 1% orcein (w/v) in 45% acetic acid (v/v) for 20 min and differentiated by gently running differentiation solution (acetic acid: distilled water: glycerol; mixed in ratio of 1:3:1), between the slide and coverslip. The orcein stained oocytes were evaluated under a phase contrast microscope at 200X and were classified into germinal vesicle (GV), intermediate and metaphase II (M-II) stage based on the nuclear maturation.

Statistical analysis

The data of in vivo matured oocytes was expressed in percent and analyzed using chi-square test by SPSS-16 Statistical program. P value of <0.05 was considered as significant.

RESULTS AND DISCUSSION

The numbers of follicles aspirated in 2-Day and 3-Day group were 60 and 89 with recovery of 38 and 50 COCs (4.75±1.08 and 6.25±1.01 per buffalo), respectively. Overall oocyte recovery rate by OPU was 58.3 percent. *In vivo* maturation of collected oocytes was assessed by degree of cumulus cell expansion and later by orcein staining.

Cumulus cell expansion: The percentage of different categories of oocyte retrieved in each group and their degree of cumulus cell expansion are presented in Table 1. Higher proportion of expanded oocytes was recovered in 3-Day protocol compared to 2 Day protocol (36.0% vs. 13.2%), whereas the proportion of compact COCs were higher in 2-Day protocol (Table 1). The higher number of expanded COCs could be due to aspiration of more number of follicles measuring >8mm in 3-Day protocol compared to number of >8mm follicles aspirated in 2-Day protocol group (5.75±1.25 vs. 1.50±0.38; P=0.006) as described by Singh *et al.* (2017).

Nuclear maturation assessment by orcein staining: Significantly (P<0.05) higher percentage of oocytes reached metaphase-II stage in 3-Day protocol compared to 2-Day protocol (40% vs.15.8%, respectively; Table 2). In a similar study on beef cows, higher percentage (58%) of matured oocytes (M-II) was retrieved using a longer FSH protocol compared to present study by Honparkhe (2011). The higher number of immature oocytes obtained in 2-Day protocol group compared to 3-Day protocol group may be due to aspiration of higher number of follicles measuring >8mm in 3-Day protocol compared to 2-Day protocol group (1.50±0.38 vs. 5.75±1.25; P=0.006).

Various factors like follicular size, follicular health, hormonal profile and ovarian status had been found influencing developmental competence of oocytes. Pavlok *et al.* (1992) investigated the capacity of the bovine oocyte derived from different follicular sizes to undergo normal fertilization and early embryonic development *in vitro* and reported higher percentages of embryonic development and blastocyst rate from oocytes recovered from the large follicles. Neglia *et al.* (2003) reported that OPU-derived buffalo oocytes have a higher developmental competence compared to abattoir-derived ones despite their worse morphological appearance. In the present study, higher *in vivo* maturation was achieved in 3 Day compared to 2 Day superstimulation protocol. Follicle stimulating hormone support is essential for the growth of growing follicles. Withdrawal of exogenous FSH support (FSH starvation) would cause smaller follicles to undergo atresia due to which a homogenous population of medium to larger follicle would be available for aspiration (Ginther *et al.,* 1996). In cattle, this arrest of gonadotropin support during superstimulation before OPU in presence of endogenous LH has been used to improve developmental competence of collected oocytes (Blondin *et al.,* 2002).

Table 1: Effect of duration of superstimulation (2 day *vs.* 3 day) on *in vivo* oocyte maturation assessed by expansion of cumulus cells.

				Cumulus cell expansion		
				Compact	Intermediate	Expanded
S. No.	Parameters	Follicles aspirated	Oocytes recovered	Number (%)	Number (%)	Number (%)
a)	2-Day protocol, without LH (n=4)	26	17	9 (52.9)	6 (35.3)	2 (11.8)
b)	2-Day protocol, with LH (n=4)	34	21	8 (38.1)	10 (47.6)	3 (14.3)
Total (a + b)		**60**	**38**	**17 (44.7)**	**16 (42.1)**	**5 (13.2)***
c)	3-Day protocol, without LH (n=4)	38	21	4 (19.0)	10 (47.6)	7 (33.3)
d)	3-Day protocol with LH (n=4)	51	29	5 (17.2)	13 (44.8)	11 (37.9)
Total (c + d)		**89**	**50**	**9 (18.0)**	**23 (46.0)**	**18 (36.0)***

(Values marked with superscript (*) in a column differ significantly at 5% level; P=0.015).

Table 2: Effect of duration of superstimulation (2 Day vs. 3 Day) on nuclear maturation of oocytes collected by OPU in buffalo.

			GV stage	Intermediate stage	M-II stage
S. No.	Parameters	Oocyte stained	Number (%)	Number (%)	Number (%)
a)	2-Day protocol without LH (n=4)	17	8 (47.0)	7 (41.2)	2 (11.8)
b)	2-Day protocol with LH (n=4)	21	9 (42.9)	8 (38.1)	4 (19.0)
Total (a+b)		**38**	**17 (44.7)**	**15 (39.5)**	**6* (15.8)**
c)	3-Day protocol without LH (n=4)	21	7 (33.3)	6 (28.6)	8 (38.1)
d)	3-Day protocol with LH (n=4)	29	8 (27.6)	9 (31.0)	12 (41.4)
Total (c+d)		**50**	**15 (28.0)**	**15 (32.0)**	**20* (40.0)**

(Values marked with superscript (*) in a column differ significantly at 5% level; P=0.013).

In the present study, the follicles measuring >6 mm of size were subjected to OPU after 48 hours of last FSH injection in both the groups. Luteinizing hormone (LH) was administered 6 hours prior OPU in half number of buffaloes to induce ovulatory LH surge. Percentage of oocytes at metaphase II stage was higher in the LH subgroups (b & d) than non-LH subgroups (a & c). The exogenous administered LH (and probably endogenous LH surge also) might have helped in germinal vesicle breakdown and progression to subsequent stages. During this period, several structural changes take place in nucleus and cytoplasm of oocyte which help the oocyte in gaining the potential required for maturation (Leibfried-Rutledge *et al.,* 1989 and Sirard *et al.,* 1989). On the contrary, *in vitro* matured oocytes would miss or bypass such ultra-structural modifications which take place in the oocyte of dominant follicle before and during LH peak (Eppig, 1996, Hyttel *et al*., 1997, Motlik and Fulka 1981). Assey *et al.* (1994) observed that bovine oocytes aspirated from dominant follicles before the LH surge display alterations in their nuclear and cytoplasmic morphology, which could be prerequisite for the acquisition of full developmental competence. Thus, indicating that processes occurring between LH surge and ovulation were also important. Therefore, *in vivo* maturation could be helpful in collecting developmentally competent oocytes for IVP in buffaloes.

CONCLUSIONS

The preliminary trial demonstrated that *in vivo* matured oocytes could be successfully collected following superstimulation in buffaloes, and 3-day superstimulatory protocol yielded higher percentage of *in vivo* matured oocytes than 2-Day protocol. Further studies are required to assess the subsequent *in vitro* developmental competence of *in vivo* matured oocytes in buffaloes.

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CONFLICT OF INTEREST

The authors declare no competing interest with this manuscript.

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