RESEARCH ARTICLE

Ovum Pick-Up and *In Vitro* Embryo Production from Stimulated *Vs* Non-Stimulated Buffaloes

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Abstract

The effect of ovarian stimulation prior to ovum pick-up (OPU) is yet to be fully assessed in buffaloes. Hence the present study was designed to assess the effect of different ovarian stimulation regimes before OPU on *in vitro* embryo production (IVEP) in buffaloes. Nine selected donor buffaloes were equally and randomly allocated to two stimulation protocols (SP1 and SP2) and a non-stimulated control. In the SP1 group, an intravaginal progesterone device (CIDR) was inserted along with Estradiol Benzoate (2.0 mg, im) on a random day of the estrous cycle (Day 0). In SP2 group, two $PGF_2\alpha$ injections were administered im 11 days apart, followed by 10 µg GnRH im on day 14. In SP1 on days 4 and 5, and in SP2 on days 16 and 17, buffaloes were administered a total of FSH 200 mg im, divided in four tapering doses at 12 hours interval (57, 57, 43, and 43 mg). In the control group, OPU sessions were performed on day 10 of $PGF_2\alpha$ induced estrus, while OPU sessions were carried out after 52 hours of coasting period in SP1 and SP2 groups. Two OPUs were carried out on each donor across experimental groups. Means of different groups were compared using one-way ANOVA using a general linear model. Although a mean number of follicles aspirated per buffalo remained similar across all groups (22.0 ± 6.9 vs. 22.0 ± 6.0, 21.2 ± 5.8; p = 0.99), a mean number of oocytes recovered/buffalo/OPU (14.8 ± 7.8 vs. 11.5 ± 3.4, 7.8 ± 2.3; p = 0.63) and oocyte recovery rate (59.2 ± 13.2 vs. 54.5 ± 4.8, 36.7 ± 3.4%; p = 0.17) was higher in the control group compared to both the stimulated groups. Attributing to ovarian stimulation in both the stimulated groups, the cleavage rate (39.2 ± 10.1 vs. 66.3 ± 7.8, 68.9 ± 10.1%; p = 0.07) and blastocyst rate (8.7 ± 5.3 vs. 32.1 ± 8.9, 17.3 ± 7.1%; p = 0.10) were higher compared to the control group. The highest embryos/OPU (1.3 ± 0.8, 3.3 ± 1.0, 1.5 ± 0.6; P = 0.23) were achieved in SP1 group as compared to Control and SP2 groups. The results indicate that ovarian stimulation increases oocyte quality, especially when stimulated in the presence of exogenous luteal support, translating into better and cost-efficient IVEP in buffaloes.

Keywords: Buffalo, CIDR, Embryo production, FSH, OPU-IVEP, Ovarian stimulation.

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Introduction

India is looked upon as an abode of best buffalo germplasm in the world. Buffalo is the most important farm animal species of Asia, especially of India, where it is extensively used for milk, meat, and draught power. It gets higher importance as buffalo account for over 49% of the total milk production of India. Buffalo has the ability to convert poor-quality roughage into milk and meat (Thanh and Orskov, 2017). Hence, the majority of the buffalo population is reared by small and marginal farmers under mixed farming conditions. Buffalo milk industry observed stunted growth for a long period in India due to its poor breeding efficiency, lack of exclusive research on buffaloes, and extrapolating findings of research in cattle onto buffalo. In addition, lack of high genetic merit proven buffalo sires for semen production challenges faster genetic improvement of buffalo via artificial insemination. There is a dire need to establish a nuclear herd of superior buffalo for genetic improvement of the species. Assisted Reproductive Technologies (ARTs) like ovum pick-up (OPU) coupled with *in vitro* embryo production (IVEP) promises faster multiplication of presently available superior bubaline germplasm for accelerated genetic improvement of the species. The key advantage of the technique is that it enables higher selection intensity and reduction in the generation interval.

It is observed that the OPU-IVEP procedures are more suited to cattle in comparison to buffalo species. Genetically, buffaloes are predisposed to smaller ovaries and poor ovarian activity as compared to cattle (Drost, 2007). There is a tenfold lower follicular reserve in buffalo as compared to cattle

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(Santos *et al.,* 2013), reflecting in a lower number of follicle recruitment in each wave of follicular growth (Campanile *et al.,* 2010) and high incidence of follicular atresia among the growing follicles in buffaloes (Palta *et al.,* 1998). Therefore, the lower number of immature oocytes recovery per donor per OPU and poor blastocyst rates following *in vitro* culture and the effect of the seasonality on oocyte quality are the major limitations of the technique in buffalo (Baruselli *et al.,* 2018). The high cost and low efficiency of IVEP in buffalo limit its efforts for wide application in the industry.

One of the possible interventions to improve the OPU efficiency could be follicular wave synchronization and stimulation by exogenous gonadotropins before OPU. Follicular wave synchronization prior to OPU was recently observed to improve the embryo production rates and the post-transfer conception rates in bovines (Cavalieri, 2018). Ovarian stimulation with exogenous hormone protocol helps recruitment of more follicles in follicular waves. Also, it increases the visibility of follicles by increasing their size during aspiration (OPU), promising higher positive end results of this technology in buffaloes. Bovines have been subjected to different types of stimulation protocol, and their response to stimulation varies from one protocol to another (Chaubal *et al.,* 2007). Likewise, similar protocols produced variable stimulation response in terms of follicles available for aspiration and oocyte recovery rate (Carvalho *et al.,* 2019). Therefore, various factors which affect the response to stimulatory treatment in terms of oocyte recovery rate and embryo production efficiency in buffaloes are yet to be elucidated. Additionally, stimulation protocols incorporate various combinations of hormones like progesterone (P_4), estrogen, prostaglandins, etc., along with gonadotropins to induce synchronized wave emergence and stimulation. This factor may contribute to the variable stimulatory response observed between donors and between protocols. Therefore, efficacies of this stimulation protocol with respect to dosage regimen and combination with different hormones are yet to be fully assessed and standardized in buffaloes. Likewise, how the estrous cycle phase during stimulation treatment initiation affects the buffalo's stimulatory response needs to be studied. The present study envisaged to evaluate and compare ovarian stimulation with exogenous FSH before OPU on total follicles aspirated (≥3 mm), total oocyte yield, oocyte recovery rate, and the rate of IVEP in buffalo donors.

Materials and Methods

The research was conducted at the Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science, AAU, Anand in collaboration with the R&D facility on OPU-IVEP, National Dairy Development Board (NDDB), Anand. The donor buffaloes included in the study were sourced from nearby farms of progressive dairy farmers of Amul milk shed area in Chikhodra village, Anand. Research work was approved by the Institutional Animal Ethics Committee (IAEC), AAU, Anand.

Animal Selection and Management

The study was conducted on Banni breed of Indian riverine buffalo (*Bubalus bubalis*) at a local organized dairy farm at Chikhodra village near Anand city, located at latitude 22° 33'54" N and longitude 73° 0'5" E with an average elevation of 35 meters above mean sea level. The research was carried out from January to April 2021, coinciding with the end of the breeding season (Nov-Feb) and increasing day length. The donor buffaloes (n = 9) selected for the study were high milk producers (12–15 lit/day), pluriparous, normal cyclic, lactating, weighing 500-600 kg, having BCS of more than 3 (scale 1–5) (Alapati *et al.,* 2010). All buffaloes were non-pregnant and free from any ovarian and uterine abnormalities. Buffaloes were maintained under a stall-fed housing system with *ad-lib* water and mineralized salt, dewormed, and vaccinated against infectious diseases regularly.

Experimental Design and Treatment Protocols (Figure 1)

The selected donors were equally (n = 3) and randomly allocated to stimulation protocol No. 1 (SP1) {CIDR + FSH}, stimulation protocol No. 2 (SP2) {GnRH + FSH} and non-stimulated group (Control). The donors were stimulated with respective stimulation protocols, and oocytes were aspirated

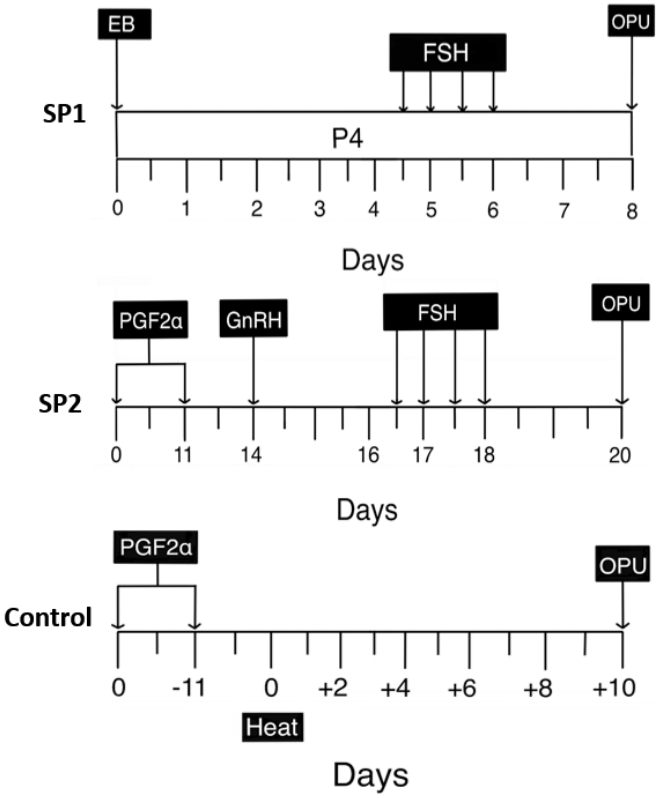

Figure 1: Schematic representation of (SP1) stimulation protocol 1, (SP2) stimulation protocol 2, and (Control) non-stimulated control

using OPU. Collected oocytes were graded and subsequently cultured in the lab for *in vitro* maturation (IVM) followed by *in vitro* fertilization (IVF), and later the presumptive zygotes were cultured *in vitro* (IVC) for the next 6 days for embryo development. Seventy-two hours post-IVF cleavage was evaluated in oocytes followed by evaluation of stage of embryo development on days 6 and 7. All donors were subjected to 2 OPU cycles, each at a gap of 1 estrous cycle across the different experimental groups.

SP1 - Stimulation Protocol No. 1 {CIDR + FSH}

Buffaloes (n = 3) received an intravaginal progesterone device (P_4: 1.38 gm; Eazi Breed™ CIDR® intravaginal implant; Boehringer Ingelheim, India) along with Estradiol Benzoate (EB) (2 mg, im) (Pregheat®, Virbac Animal Health, India) at the random stage of the estrous cycle (day 0). From day 4 onwards, buffaloes were stimulated using FSH 200 mg (Folltropin®-V, Vetoquinol N.A. Inc, Canada), divided into four tapering doses (57 mg, 57 mg, 43 mg, 43 mg, respectively, im) given at 12 hours intervals. On Day 8 (52 hr of "coasting" period), OPU was performed along with removal of P_4 device (CIDR).

SP2 - Stimulation Protocol No. 2 {GnRH + FSH}

Buffaloes (n = 3) were synchronized by two $PGF_2\alpha$ administrations 500 µg each, im (Cloprostenol sodium, Estrumate®, Vet Pharma Friesoy the GmbH, Germany) 11 days apart with the first $PGF_2\alpha$ on a random day of the estrous cycle. On day 14, buffaloes were administered with 10 µg GnRH analog im (Buserelin acetate, Receptal® VET, Intervet International GmbH, Germany). On day 16 onwards, buffaloes were stimulated using FSH 200 mg (Folltropin®-V, Vetoquinol N.A. Inc, Canada), divided into four tapering doses (57, 57, 43, 43 mg, respectively im) given at 12 hourly intervals. OPU was performed after 52 hours of last FSH administration.

Control Group

Buffaloes under the control group (n = 3) were synchronized by administering with two $PGF_2\alpha$ injections 500 µg each, im (Cloprostenol sodium, Estrumate®, Vet Pharma Friesoythe GmbH, Germany) 11 days apart and the heat was detected on day $14^{th,}$ and OPU was performed on day 10^{th} post-estrus.

Ovum Pick-Up and *In Vitro* Embryo Production (OPU-IVEP)

The OPU-IVEP in the donor buffaloes was performed using an already standardized procedure (Patel, 2020). Briefly, the donor buffaloes were aspirated using an ultrasound-guided transvaginal aspiration device under epidural anesthesia. The ovaries were evaluated for the number of follicles and corpus luteum in both stimulated and non-stimulated donors. All follicles of 3 mm or more were aspirated into a 50 mL collection tube having OPU media. The same operator performed all oocyte recovery procedures. After aspiration of both ovaries, the collection tube was immediately sealed and sent inside a temperature-controlled (38.5°C) Styrofoambox to a laboratory located ~7 Km away from the OPU site for oocyte searching and further processing. Immature oocytes were matured, fertilized, and cultured *in vitro*. Maturation was performed for 22 hours in a CO_2 incubator (38.5°C, 5% CO2 in air in maximum humidity) followed by fertilization for 18 hours in a CO_2 incubator (38.5°C, 5% CO_2 in air in maximum humidity) and thereafter, the presumtive zygotes were transferred in culture media (up to 7 days after fertilization, in benchtop incubator at 38.5°C, 5% CO_2, 5% O_2 in maximum humidity). Embryo development was assessed for cleavage at 72 hours after fertilization and embryo development (stage and quality grades according to standardized protocol) at day 6 and 7 after fertilization. Blastocyst rate was calculated by dividing the total number of blastocysts by a total number of oocytes kept for culture.

All the media used for OPU-IVEP were procured from IVF Bioscience, UK. Sperm separation medium was procured from FUJIFILM Irvine Scientific.

Statistical Analysis

Descriptive statistics were used to calculate OPU-IVEP parameters in SP1, SP2, and ocntrol group. The values were expressed as Mean ± SEM and were compared between different groups (SP1, SP2, and Control) using one-way ANOVA using the general linear model on SigmaPlot 11 ® software (Systat Software Inc., USA). Percentage values were subjected to Arcsine transformation before comparing through ANOVA. Means were considered to be statistically different when $p < 0.05$.

Results and Discussion

The effects of ovarian stimulatory treatment on OPU-IVEP parameters are summarized in Table 1. Ovarian stimulatory treatment had no effect on the number of follicles available for aspiration during OPU (SP1 = 22.0 ± 6.0; SP2 = 21.2 ± 5.8; Control = 22.0 ± 6.9; p = 0.99), whereas the number of oocytes recovered per buffalo per OPU session (p = 0.63) and oocyte recovery rate per OPU session (p = 0.17) were higher in the control group as compared to both the stimulated groups. Stimulation did not affect the grade of oocyte recovered; the majority percentage of collected oocytes were categorized under Grade III (p = 0.16) or Grade IV (p = 0.61) across all groups. Both the buffalo donor groups subjected to respective ovarian stimulation protocol produced a higher cleavage rate (p = 0.07) and blastocyst rate (p = 0.10) as compared to the control group. Additionally, the SP1 group of buffaloes produced a higher blastocyst rate and embryo per OPU-IVEP session (p = 0.23) than the SP2 and control group.

It was found that ovarian stimulation had no effect on the number of follicles available for aspiration. Consequently, the oocyte recovery rate decreased in stimulated groups, but the resulting blastocyst rate was greater than non-stimulated

Table 1: Summary of OPU-IVEP responses (mean ± SEM) of buffalo donors subjected to ovarian stimulation prior to OPU

	Animal groups			
Parameters	*Non-stimulated {Control}*	*Stimulation I (SP 1) {CIDR + FSH}*	*Stimulation II (SP 2) {GnRH + FSH}*	*p-Value*
Number of OPUs	06	06	06	--
Total follicles aspirated	132	132	127	--
Mean follicles aspirated	22.0 ± 6.9	22.0 ± 6.0	21.2 ± 5.8	p = 0.99
Total oocytes recovered	89	69	47	--
Mean oocytes recovered	14.8 ± 7.8	11.5 ± 3.4	7.8 ± 2.3	p = 0.63
Oocyte recovery rate (%)	59.2 ± 13.2	54.5 ± 4.8	36.7 ± 3.4	p = 0.17
Mean cleavage rate (%)	39.2 ± 10.1	66.3 ± 7.8	68.9 ± 10.1	p = 0.07
Mean no. of embryo/OPU	1.5 ± 0.8	3.3 ± 1.0	1.5 ± 0.6	p = 0.23
Blastocyst rate (%)	8.7 ± 5.3	32.1 ± 8.9	17.3 ± 7.1	p = 0.10

donors, indicating an increase in oocyte quality attributed to ovarian stimulation. The present study supports the hypothesis that ovarian stimulation prior to OPU increases IVEP response in buffaloes.

In fact, it was well documented in previous studies and also observed during the present study that under the influence of exogenous FSH, there is an increase in the size of the antral follicles on the ovaries of stimulated buffaloes and HF cattle (medium to large-sized follicle) than that observed on the ovaries of non-stimulated animals (Presicce *et al.,* 1997; Vieira *et al.,* 2014; Carvalho *et al.,* 2019). Also, mean oocytes recovered, and oocyte recovery rate were higher in non-stimulated donors than in stimulated donors. However, these reports had improved blastocyst rate and embryos per OPU in stimulated donors as compared to control. Additionally, the oocyte recovery from smaller follicles was considerably better than larger follicles as there was fewer liquor folliculi, less intra-follicular pressure, and viscosity than medium or large follicles (Seneda *et al.,* 2001). Therefore, such medium or large follicles tended to burst or collapse due to higher intra-follicular pressure when punctured during aspiration losing the cumulus oophorus complex, affecting the oocyte recovery and recovery rates.

In non-stimulated buffaloes, most oocytes were recovered from smaller follicles, resulting in high oocyte recovery (Seneda *et al.,* 2001). Nevertheless, these follicles might be in a different stage of growth and coupled with a high incidence of atresia among growing follicles in buffalo (Danell, 1987) as developmental competence of recovered oocyte was poor. Hence the rate of embryo production was lowest in non-stimulated buffaloes. COCs recovered from follicles with larger diameters have greater developmental competence when compared to COCs from smaller follicles (Lonergan *et al.,* 1994). Therefore, these results support that the increase in follicular diameter caused by FSH treatment may improve oocyte quality, resulting in greater IVEP efficiency.

Ovarian stimulation also had no effect on the grade of oocyte recovered, as none of oocytes collected was under Grade I and II category, irrespective of stimulation or non-stimulated groups. The majority of collected oocytes fell under Grade III and Grade IV categories across all the groups. In accordance with our findings, Sagheer *et al.* (2020) performed OPU in Nili-Ravi buffalo during peak breeding season and observed that among the oocytes collected, the proportion of grade III and grade IV was higher as compared to grade I and grade II.

Lower-grade of oocytes is contributed by multiple factors ranging from aspiration pump pressure, breed variation, technician skill and handling by laboratory technician after aspiration, etc. However, it was observed by Neglia *et al.* (2003) that despite of lower grade of OPU derived oocytes, these oocytes have higher developmental competence than oocytes derived from slaughtered ovaries. Also, the lower grade oocyte may be of good quality as the grading of oocytes is highly dependent on a number of cumulus cell layers which can wear off during aspiration and post-aspiration handling by technicians (Patel, 2020). Therefore, high chances are there that Grade III and Grade IV oocytes recovered through OPU may convert in to good quality blastocysts. Therefore, oocytes falling under lower grades, *i.e.,* Grade III and Grade IV are considered culturable and were subjected to IVEP protocols.

Indeed, in the present study, ovarian stimulation had a positive impact on rate of embryo production in buffaloes. Further, among the two stimulation protocols implemented for ovarian stimulation, protocol 1{CIDR + FSH} appeared better than stimulation protocol 2 {GnRH + FSH} in almost all aspects. The possible reason for the better result of CIDR + FSH protocol is the controlled emergence of the follicular wave subsequent to stimulation. A new follicular wave emerges four days post-administration of oestradiol (Honparkhe *et al.,* 2014). This rendered a pool of uniform growing follicles for FSH stimulation. The exogenous FSH superimposed the endogenous FSH surge and stimulated follicular growth. Additionally, the exogenous P_4 support (CIDR) had a facilitatory effect that helps reduce the diameter

Table 2: Comparison of cost-effectiveness between both the stimulated and non-stimulated OPU-IVEP cycles

Consumables	*Cost per unit* (INR)*	*Control*	*SP1*	*SP2*
OPU-IVEP cycles	12,500.00**	06	06	06
CIDR	800.00	--	06	--
Estradiol Benzoate (Pregheat®)	180.00		06	--
FSH (Folltropin®-V)	11000.00	--	3 Vials	3 Vials
GnRH (Receptal®)	810.00	--	--	1.5 Vial
$PGF_2\alpha$ (Estrumate®)	1690.00	1 Vial	--	1 Vial
Total cost (INR)		76,692.00	113,880.00	110,905.00
Total embryos produced	--	08	20	09
Cost per embryo produced (INR)	--	9,586.00	5,694.00	12,322.00

(*Costs mentioned above are subjected to vary in different regions, ** includes the cost of consumables and media cost used per OPU-IVEP cycle.)

of larger follicles while accumulating medium follicles by reducing the rate of growth and atresia of the follicles (Hafez and Hafez, 2000). Better *in vitro* developmental competence and higher fertilization rates are obtained from oocytes recovered from medium to large follicles with higher P_4 levels (Lonergan *et al.,* 1994; Lamb *et al.,* 2010). Therefore, the present study supports positive role of exogenous P_4 support (CIDR) in improving oocyte quality. Overall better rate of embryo production was observed when stimulation was done under the influence of exogenous P_4 support in buffaloes (Carvalho *et al.,* 2019) and in cattle (Vieira *et al.,* 2014; Vieira *et al.,* 2015) as compared to non-stimulated donors. Contrastingly, better oocyte recovery rates and better oocyte quality have been obtained from follicles with relatively low circulating P_4 concentrations (Abreu *et al.,* 2018). Therefore, it requires further studies to evaluate the effect and determine the optimal dose of P_4 on oocyte quality and devise a strategy for using this substance prior to the OPU procedure.

On the other hand, in SP2 (GnRH + FSH) protocol, estrus synchronization by two PGF2α administrations 11 days apart failed to synchronize estrus due to its variable response in buffaloes tightly, and GnRH proved inefficient in synchronizing ovulation and induction of a new follicular wave. Even though exogenous FSH induced follicular growth, the growth of follicles was not homogenous, probably due to the absence or lower concentration of circulating P_4 due to developing early corpus luteum. In addition, a low number of oocytes were recovered in the SP2 group, resulting in a poor rate of embryo production compared to CIDR + FSH protocol. Subsequently, there is considerable variation in individual donors' response to ovarian stimulation and subsequent IVEP procedures. Few donors in all the groups responded excellently to stimulation and embryo production, while others failed to reflect the same. Marin *et al.* (2019) increased mean embryo production by 0.7/animal/OPU to 1.2/animal/OPU by selecting donors regarding their mean rate of embryo production. Therefore, selecting individual donors based on their mean blastocyst production rates could be a valid strategy to increase embryo production rates by IVF, thereby making OPU-IVEP an affordable tool for genetic improvement programs in the buffalo species.

To envisage practical applicability of present study, cost-effectiveness of both the stimulation protocols against non-stimulated control was worked out based on the results obtained and considering market prices of different materials and hormones used in the study (Table 2). Although stimulation protocol SP1 (*i.e.*, CIDR + FSH) had the highest input cost compared to other groups, the cost per embryo produced was lowest among all groups.

Conclusion

The present study results indicated that ovarian stimulation in buffaloes prior to OPU increases IVEP efficiency by increasing follicular size and not by increasing follicular population. Ovarian stimulation under the influence of exogenous luteal support facilitates homogenous follicular growth by preventing the development of atretic changes and thereby improving oocyte quality. Ovarian stimulation with CIDR and FSH promises efficient and cost-effective OPU-IVEP in buffaloes. However, the data obtained in the present study is very less due to a smaller sample of animals subjected to the study to conclude statistically significant results. It necessitates further experimentation on a large sample size to find out the most effective protocol for efficient OPU-IVEP in buffalo.

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