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Effect of FSH Stimulation of Ovaries on *in vitro* Maturation of Sahiwal Oocytes Collected by Ovum Pick-up (OPU) Method

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ABSTRACT

Background: For conservation of breeds and faster multiplication of superior germplasm in a short period, ovum pick up-*In vitro* embryo production (OPU-IVEP) in combination with embryo transfer (ET) can be a viable alternative to the multiple ovulation embryo transfer (MOET). The aim of the present study was to evaluate the effect of FSH stimulation on follicular population, oocyte recovery rate and *in vitro* maturation of oocytes collected by ovum pick-up in Sahiwal cows.

Methods: Sixteen Sahiwal cows aged 3-6 years were randomly divided into two groups. Animals in group 1 (Non-stimulated, n=8) were subjected to ovum pick-up (OPU) twice at 96h interval at random stage of estrous cycle. Animals in group 2 (FSH stimulated, n=8) were subjected to FSH prestimulation (Follitropin-V, 200 mg i/m in 3 tapering doses 100, 60, 40 mg) prior to OPU 1 and with a duration of 96 hrs interval OPU 2 performed.

Result: There was significant difference ($p<0.05$) observed between FSH stimulated and non-stimulated group in mean number of follicles available for aspiration (15.31±1.56 and 6.88±0.55, respectively), mean number of oocytes recovered (4.94±0.70 and 3.25±0.40, respectively), cumulus cell expansion rate (92.95 and 83.7% respectively) and 1st polar body extrusion rate (74.64 and 67.44%, respectively). In conclusion, 200 mg of FSH stimulation with decreasing dose prior to OPU increased the follicular population and increased more number of medium and large follicles available for aspiration. FSH pre-treatment also improved the mean number of oocyte recovery and maturation rate compared to non-stimulated group.

Key words: Cumulus cell expansion, Follicle stimulating hormone, *In vitro* maturation, Ovum pick-up (OPU), Polar body extrusion.

INTRODUCTION

Among the cattle breeds of India, Sahiwal cow is well known for its unique traits such as disease resistance, parasitic resistance, heat tolerance and high lactation yield. Use of OPU in IVEP programmes proved to be a potential alternative to traditional embryo production (Bousquet *et al.*, 1999 and Kruip *et al.*, 1991). OPU and ET technology has many advantages over embryo transfer as the repeated collection of oocytes from an adult cattle may get as many as 1000 oocytes or 300 embryos/year/cow and circumvents many problems associated with the uterine environment (Hasler *et al.*, 1995 and Looney *et al.*, 1994).

Ovum pick-up can be performed in nearly all animals which are cyclic, noncyclic, early pregnancy (upto 3 months), those not responding to hormonal stimulation, older cows with reproductive disorders, juvenile calves and prepubertal heifers (Presicce *et al.*, 1997; Majerus *et al.*, 1999 and Taneja *et al.*, 2000). Repeated aspirations at short time intervals are possible and the oocytes can be recovered from cows irrespective of their reproductive phase (Bungartz *et al.*, 1995).

Repeated aspirations can be performed twice weekly for several months without need for hormonal stimulation of donors. Stimulation with FSH prior to OPU increase oocyte recovery rate compare to once in a week non-stimulated OPU procedure (Chaubal *et al.*, 2006). Aspiration technique, aspiration pressure, needle type, coasting period also play a major role and animal factors include Breed, age, stage of lactation, nutrition and seasonal conditions also influence the oocyte recovery rate.

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Moreover OPU is the only means of obtaining oocytes from live cattle in India where cow slaughter is not allowed due to religious reasons. The present study was therefore, aimed to standardize an efficient ovum pick-up (OPU) protocol in Sahiwal cows with the aim to compare oocyte recovery and *in vitro* maturation rates of oocytes collected by OPU in stimulated and non-stimulated Sahiwal cows.

MATERIALS AND METHODS

Experimental location

The present study was undertaken at Livestock Farm Complex, College of Veterinary Science, Korutla, Jagitial district, Telangana (latitude: 18°49′36.71″N; longitude:

78°42′50.39″E; altitude: 295.99 m above mean sea level) during the period between August and December, 2020.

Experimental animals

Sixteen Sahiwal cows (*Bos indicus*) aged 3-6 years and weighing between 250 and 450 kg body weight were selected as oocyte donors and randomly divided into two groups. The daily ration of each animal consists of 2-3 kg high protein feed containing 20% DCP and 70% TDN, 8-12 kg chopped green fodder and 3-5 kg paddy straw provided with ad libitum drinking water. Animals were maintained under hygienic and optimum management conditions in loose housing system with a large, open paddock for free movement. Health and vaccination protocols were followed as per standard schedule.

Superstimulation protocol and OPU schedule

Irrespective of the stage of estrus cycle, the cows were randomly divided into two groups. Cows in group 1 (non-stimulated, n=8) were subjected to ovum pick-up (OPU) twice at 96 hrs interval at random stage of estrous cycle. Whereas cows in group 2 (Stimulated, n=8) were administered 10 µg Gonadotropin Releasing Hormone (GnRH) (Receptal, MSD Animal Health, 2.5 ml i/m) at random stage of estrous cycle. FSH stimulation (Follitropin-V, 200 mg i/m in 3 divided doses- 100, 60 and 40 mg); first FSH injection (100 mg) was given at 48 hrs after GnRH administration. Second FSH dose (60 mg) was given 15 hrs after first injection and third FSH dose (40 mg) was given 24 hrs after the second FSH dose. Ovum pickup was carried out 52 hrs after the last FSH injection (coasting period) and 2nd OPU was carried out with an interval of 96 hrs after OPU-1. A total of 32 OPU sessions were performed in 16 cows with and without FSH prestimulation.

Follicle aspiration and oocyte recovery

Following caudal epidural anesthesia the ovary was manipulated gently and positioned against the probe head in order to obtain a clear image of the follicles on the ultra-sonographic monitor (Fig 1). The number of follicles per ovary was recorded and the diameter of the follicles was measured by freezing the image on the monitor and by using an inbuilt caliper (Fig 2 and 3) and the follicles were classified as small (<4 mm), medium (4-<8 mm) and large follicles (≥8 mm) (Ginther *et al.*, 1989). During each aspiration, all visible follicles of were aspirated and the contents were collected in a 50 ml tube. The oocyte recovery rate was calculated as the number of oocytes recovered from the number of follicles aspirated for each cow expressed as a percentage (Goodhand *et al.*, 2000).

The tube containing the follicular aspirate was transferred to a 75 µ cup filter (Emcon Immunosystems Inc., Biddeford, USA) and repeatedly washed with Euroflush medium (catalog no. 019450, IMV technologies, USA) in order to make the filtered aspirate free from blood tinge and cloudy follicular fluid. The washed and filtered follicular aspirate was then transferred to a square grid petridish 90×15 mm (Catalog no. 150360, Thermo scientific, Massachusetts, USA) and examined under stereozoom microscope (SMZ-1270, Nikon, Japan) at 1x magnification to identify the cumulus oocyte complexes (COCs). The COCs were transferred to a 35 mm petridish containing BO-Wash medium (Catalog no. 51002, IVF Biosciences, Denmark) and examined under stereozoom microscope at 8x magnification for evaluation and grading.

Evaluation of cumulus oocyte complexes

In this study, the cumulus oocyte complexes were classified into four quality grades (Grade 1, Grade 2, Grade 3 and



Fig 1: OPU collection unit (USG scanner, transvaginal probe and Suction pump).

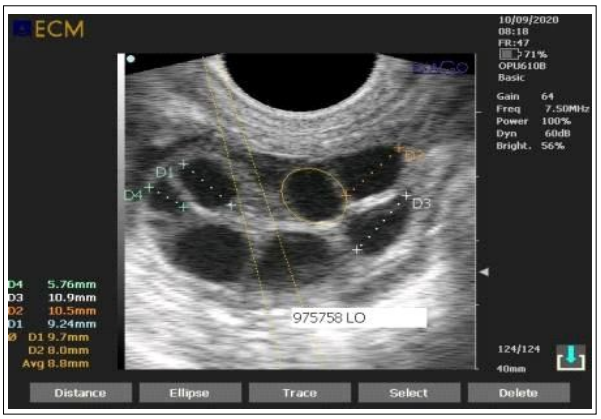

Fig 2: FSH stimulated ovary showing follicles of various size.

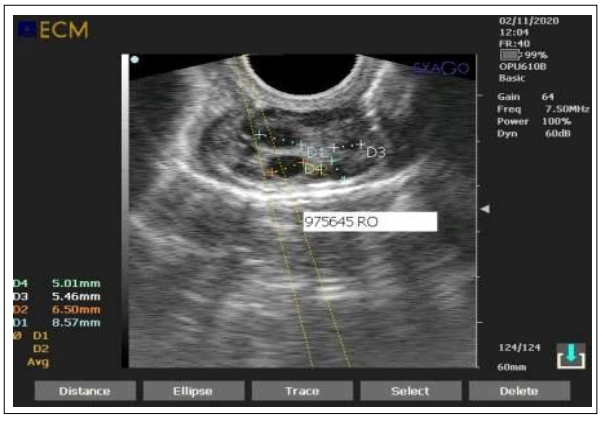

Fig 3: Non-stimulated ovary showing follicles of various size.

Grade 4) based on cumulus cells surrounded and cytoplasm (Pontes *et al.*, 2011 and Vieira *et al.*, 2016). Grade 1 - More than 3 layers of compact cumulus cells, Grade 2 - At least one layer of cumulus cells, Grade 3 - Denuded and Grade 4 -Atretic with dark cumulus cells and signs of cytoplasmic degeneration (Fig 4, 5 and 6).

All the visible follicles were aspirated in each OPU session in both groups of cows and distribution of small (<4mm), medium (4-<8 mm) and large follicles (≥8 mm), oocyte recovery rate and oocyte quality were observed and recorded for individual cows.

In vitro maturation (IVM) of oocytes

The oocytes were washed 4-6 times with BO-WASH medium (Catalog no.51002, IVF Biosciences, Denmark) and then washed twice with BO-IVM (Catalog no.71001, IVF Biosciences, Denmark). For IVM, group of 5 to 10 Cumulus-oocyte complexes were placed in 50 µl droplets of IVM medium, overlaid with sterile mineral oil in 35 mm petridish equilibrated prior to transfer of cumulus oocyte complexes and cultured in a humidified CO_2 incubator (5% CO_2 in air) at 38.5°C for 24 hrs. After 24 hrs assessment of oocyte maturation was done by the degree of expansion of cumulus cell mass and extrusion of first polar body into the perivitelline space (Fig 7 and 8).

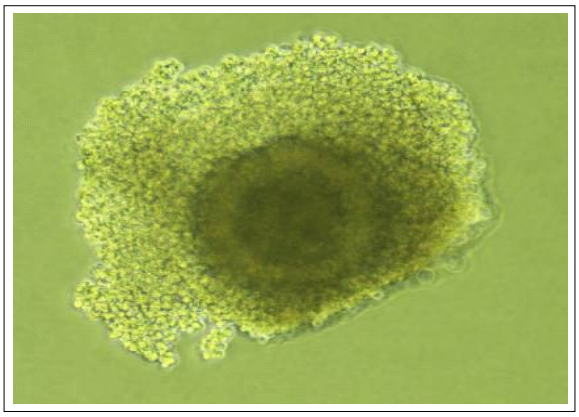

Fig 4: Grade 1 quality oocyte with more than 3 layers of cumulus cells (under 20X magnification).

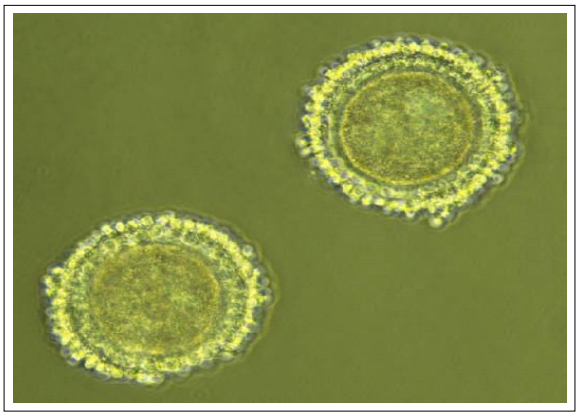

Fig 5: Grade 2 quality oocyte with one layer of cumulus cells (under 20X magnification).

RESULTS AND DISCUSSION

In the present study the mean number of follicles available for aspiration in two OPU sessions was significantly ($p<0.05$) higher in FSH stimulated group (15.31±1.56) compared to the non-stimulated group (6.88±0.55). The mean number of follicles available for aspiration in both OPU sessions were significantly higher in FSH stimulated group (OPU-1: 18.25 ±2.48 and OPU-2: 12.38±1.35) than non-stimulated group

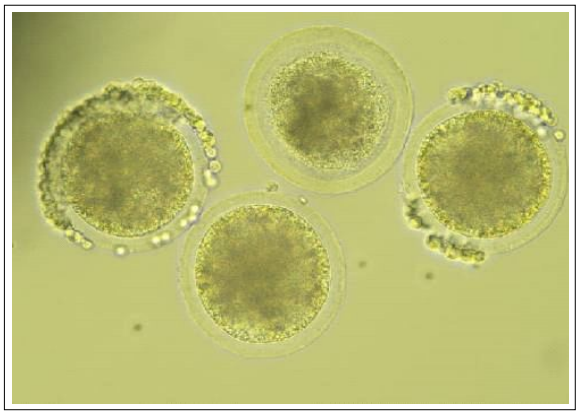

Fig 6: Grade 3 denuded (A,A) and grade 4 degenerated (B,B) oocytes (at 20X).

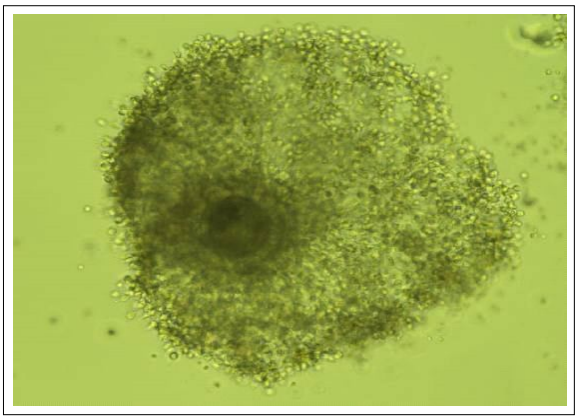

Fig 7: Grade 1 oocyte showing cumulus cell expansion 24 hrs after IVM (under 20X magnification).

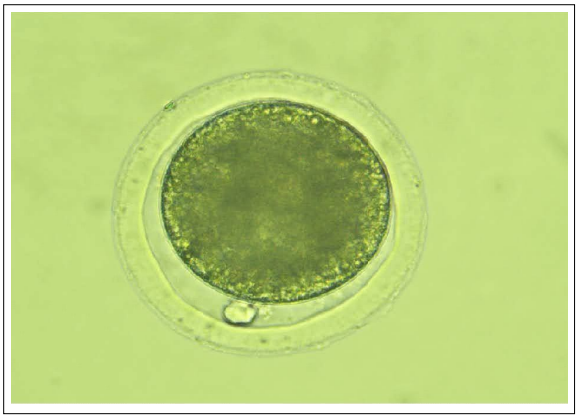

Fig 8: Grade 1 oocyte showing extrusion of first polar body (under 40X magnification).

(OPU-1: 7.50±0.87 and OPU-2: 6.25±0.65). However the number of follicles available for aspiration for OPU-1 was significantly higher than OPU-2 in FSH stimulated group (Table 1). Higher number of follicles observed in cows treated with FSH than cows without FSH treatment may be due to increased recruitment of follicles by initiating growth of more number of follicles by FSH. Though the results were similar but the mean number of follicles available in FSH treated and in non-treated group in beef × Friesian cows (Goodhand *et al.*, 2000), beef cows (Aller *et al.*, 2010) and Ongole cows (Srimannarayna, 2019) were higher than the Sahiwal cows in the present study, this may be due to the time interval between the two OPU session was shorter (96 h), variation in the dose of FSH and the different breed of cattle.

The mean number of small follicles were significantly ($p<0.05$) higher in non-stimulated group (3.63±0.34) compared to FSH stimulated group (2.25±0.36). The mean number of medium and large follicles significantly ($p< 0.05$) higher in FSH stimulated group (7.18±0.64 and 5.94±1.0) compared to non-stimulated group (2.19±0.34 and 1.25±0.32) (Table 2). Present results are in accordance with the studies on beef cows (Goodhand *et al.,* 2000; Jeyakumar, 2004), HF cows (Vieira *et al.,* 2014; Silva *et al.,* 2017; Ongaratto *et al.,* 2020) and in Ongole cattle (Sriman narayana, 2019). It is known that FSH causes the stimulation of follicular growth that caused the increased number of medium and large follicles available for aspiration in FSH stimulated group.

In the present study the mean number of oocytes recovered per session per animal was significantly ($p<0.05$) higher in FSH stimulated group (4.94±0.70) compared to the non-stimulated group (3.25±0.40). But the recovery rate was lower in FSH stimulated group (32.24%) compare to non-stimulated group (47.27%) (Table 3). Similarly in HF cows the oocyte recovery rate was low in FSH treated group (60%) than in without FSH stimulation (75%) group (Ongaratto *et al.* 2020). The number oocytes recovered in FSH treated group in the present study were comparable to the earlier studies on HF cows (Jeyakumar, 2004; Aller *et al.,* 2010; Vieira *et al.,* 2016; Silva *et al.*, 2017; Srimannarayana, 2019; Ongaratto *et al.,* 2020). The number oocytes recovered in the present study was lower than the recovery rates of the Ongole cattle (Srimannarayana, 2019). Oocyte recovery rate was higher in follicles size ≤4 mm diameter compare to >4 mm diameter (Lonergan *et al.* 1994; Seneda *et al.*, 2001). Similarly in the present study due to presence of more number of medium and large follicles in FSH stimulated group, which contains more viscous follicular fluid because corresponding oocytes are likely to be more mature and the aspirate may contain sheets of granulosa cells. The losses during aspiration would be reduced in small follicles associated with lower intrafollicular pressure with a smaller amount of intra follicular material (follicular fluid and oocyte). In FSH stimulated group the mean number of oocytes recovered from OPU 1 (6.13±0.79) was significantly ($p<0.05$) higher than OPU 2 (3.75±1.03) (Table 3). This may be due to the OPU sessions performed with short duration interval, there may be formation of residual follicles filled with blood and due to similar echogenic characters appeared as normal follicles which leads to less recovery of oocytes and increased risk of blood clots in the needle and tubing system (Petyim *et al.,* 2000).

The mean number of Grade 1, Grade 2, Grade 3 and Grade 4 oocytes recovered in non-stimulated group were 0.81±0.30, 0.68±0.17, 1.18±0.34 and 0.60±0.16 and in FSH stimulated group 1.87±0.45, 0.93±0.28, 1.43±0.27 and 0.56±0.20 respectively (Table 4). It has been reported that there was no significant improvement ($p>0.05$) on oocyte quality in cows with FSH pre-treatment similar with the results of Silva *et al.* (2017). This may be attributed to transducer type, puncture frequency, combination of needle gauge, vacuum pressure and the diameter of the follicle. There is a possibility of the oocytes aspirated from the small follicles

Table 1: Mean follicular population available for aspiration per cow per session in non-stimulated and FSH stimulated group of cows.

	Non-stimulated group mean follicular population (Total follicles)	FSH stimulated group mean follicular population (Total follicles)
OPU 1	$7.50±0.87^{a}$ (60)	$18.25±2.48^{bA}$ (50)
OPU 2	$6.25±0.65^{a}$ (146)	$12.38±1.35^{bB}$ (99)
Total (OPU-1+OPU-2)	$6.88±0.55^{a}$ (119)	$15.31±1.56^{b}$ (245)

Values bearing different superscripts within a row (a, b) and within a column (A, B) differ significantly ($p<0.05$).

Table 2: Follicle size distribution in in non-stimulated and FSH stimulated group of cows.

Follicular size	Mean number of follicles (Non-stimulated)	Mean number of follicles (FSH stimulated)
Small (<4 mm)	$3.63±0.34^{a}$	$2.25±0.36^{b}$
Medium (4-8 mm)	$2.19±0.34^{a}$	$7.18±0.64^{b}$
Large (>8 mm)	$1.25±0.32^{a}$	$5.94±1.0^{b}$

Values bearing different superscripts (a,b) within a row differ significantly ($p<0.05$).

Table 3: Oocyte recovery per cow per session in non-stimulated and FSH stimulated cows.

	Non-stimulated group Mean number of oocytes (recovery rate)	FSH stimulated group Mean number of oocytes (recovery rate)
OPU 1	$3.50±0.33^{a}$	$6.13±0.79^{bA}$
OPU 2	3.0±0.76	$3.75±1.03^{B}$
Total (OPU-1 +OPU-2)	$3.25±0.40^{b}$ (47.27%)	$4.94±0.70^{a}$ (32.24%)

Values bearing different superscripts within a row (a, b) and within a column (A, B) differ significantly ($p<0.05$).

Table 4: Oocyte quality in non-stimulated and FSH stimulated cows.

Attribute	Mean number of oocytes (Non-stimulated group)	Mean number of oocytes (FSH stimulated group)
Grade 1	0.81±0.30	1.87±0.45
Grade 2	0.68±0.17	0.93±0.28
Grade 3	1.18±0.34	1.43±0.27
Grade 4	0.60±0.16	0.56±0.20

Table 5: Effect of FSH stimulation on cumulus cell expansion and 1st polar body extrusion of oocytes.

	Non-stimulated	FSH stimulated
Number of COC's collected	52	79
COC's subjected for Maturation	43	71
Cumulus cell expansion	36	66
Cumulus cell expansion rate (%)	83.7	92.95
1st polar body extrusion	29	53
1st polar body extrusion rate (%)	67.44	74.64

have less cumulus investment, that effect the quality of oocytes. In contrast FSH stimulation prior to OPU improved the quality oocytes (Loony *et al.,* 1994; Jeyakumar, 2004; Aller *et al.,* 2010).

The cumulus cell expansion rate (92.95 vs 83.7) and first polar body extrusion rate 74.64 vs 67.44) were comparatively higher in FSH stimulated group than non stimulated group but there was no significance difference observed between groups (Table 5). Verma, (2005) assessed the oocyte maturation rate in Sahiwal cows based on degree of cumulus cell expansion and polar body extrusion and reported that there was no significance difference observed between FSH stimulated group (87.8%) and non-stimulated weekly twice OPU group (84.69%). Similarly Ratto *et al.* (2011) also reported that maturation rate in weekly twice OPU in Aberden angus cows was 70.1%. The oocytes from non-stimulated group may acquire necessary factors for cumulus expansion and further acquisition of meiotic competency during in vitro maturation. FSH stimulation prior to OPU is significantly increased the number of follicles available for aspiration and oocyte recovery rate but the quality of the recovered oocytes and their ability to exhibit cumulus expansion and extrusion of 1st polar body was not improved.

CONCLUSION

The success of OPU- IVM-IVF- ET influenced by the various factors which are related to animal: breed, age, cyclicity and nutrition of the animal; technical; vaccum pressure, diameter of the aspiration needle and skill of the operator. Furthermore the inherent ability of individual animal that supports the growth of the healthy follicles and the growth of the follicles in response to external hormones also effect on the outcome of the OPU. In conclusion, 200 mg of FSH stimulation with decreasing dose prior to OPU increased the follicular population and increased more number of medium and large follicles available for aspiration. FSH pre-treatment also improved the mean number of oocyte recovery and maturation rate including cumulus cell expansion and 1st polar body extrusion. However further studies are needed to improve the oocyte yield and oocyte maturation rate through OPUand IVM-IVF technique in native cattle breeds.

Conflict of interest

All authors declared that they have no conflict of interest.

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