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RESEARCH ARTICLE

Ovarian Stimulation through FSH Improves Follicular Harvesting and Blastocyst Yield in *Bos Indicus* Cattle

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A B S T R A C T

The present study was designed to transform small-sized follicles into medium sized follicles to enhance their developmental competence under the effect of FSH. Thirty Sahiwal cows were randomly assigned to three groups (n=10 each); A) FSH-00mg (control), B) FSH-100mg (F100), and C) FSH-200mg (F200). On a random day of the estrus cycle (-48h), each cow underwent follicular ablation and received an intra-vaginal CIDR device. After 48h (0 hour), each cow received 150µg of D-cloprostenol; while four equivalent doses of FSH (25 mg in F100 group and 50 mg in F200 group) were administered at 12h intervals (0, 12, 24 and 36 hr). Ovarian ultrasonography was performed on daily basis and Ovum Pick-Up (OPU) was conducted after 40h of last FSH treatment in groups B and C, while 76 hrs after D-cloprostenol injection in control group A. The results revealed that the number of medium sized (6≥10 mm) follicles were significantly ($P<0.05$) increased in FSH treated (F100 & F200) groups than control, while there was non-significant difference between F100 and F200 groups. During IVEP, the blastocysts yield per OPU session was almost double in F100 (2.00±0.69) and F200 (1.80±0.59) groups than the control (0.90±0.37) group. The *in-vitro* cultured blastocysts were transferred to four synchronized heifers; three heifers were pregnant and gave birth to four healthy calves, including one twin birth. To the best of our knowledge, this is the first report on the birth of genetically tested Sahiwal calves through OPU-IVEP technique in Pakistan, following fertilization of oocytes retrieved from Sahiwal cows with semen from a Sahiwal bull. In conclusion, ovarian stimulation with FSH hormone improves the proportionality of medium-sized follicles during OPU with better oocyte competence in Sahiwal cattle.

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INTRODUCTION

The global demand for *in-vitro* embryo production (IVEP) has increased tremendously over the last decade, primarily due to its high effectiveness for genetic improvement in dairy herds and better understanding of its full potential by the end-users. According to a report of the International Embryo Technology Society (IETS), the total number of *in-vitro* produced embryos has surpassed Multiple Ovulation and Embryo Transfer (MOET) for the first time in recorded history (Viana, 2018). Along with the provision of many embryos from a single animal at one estrus, IVEP allows utilizing the latest technologies of sex-sorted semen, genomic selection, and a possibility to cryopreserve the embryos for future use (Moore and Hasler, 2017).

The outcomes of an IVEP program are largely dependent on the number and size of antral follicles available at the time of ovum pick-up (OPU) (Singh *et al*., 2004). As the size of the follicle increases and approaches ovulation, developmental competence of the oocyte growing within the follicle also increases (Caixeta *et al*., 2009; Sirard, 2011; Sirard, 2012). Additionally, the oocytes retrieved from medium-sized follicles (6-10 mm) showed better *in-vitro* developmental competence than those collected from small-sized follicles in *Bos indicus*

cows (Lonergan *et al*., 1994; Sarwar *et al*., 2020a). A growing bovine oocyte houses mRNA and proteins (Gandolfi and Gandolfi, 2001), which are crucial for early embryonic development (Dieleman *et al*., 2002). Therefore, a homogenous population of medium-sized (6–10 mm) follicles would be an ideal sight on the day of OPU. In this context, several protocols for follicular wave synchronization and super-stimulation before OPU have been devised to improve the efficiency of IVEP programs (Seneda *et al*., 2020).

Super-stimulation prior to OPU with either equine chorionic gonadotropin (eCG) or follicle stimulating hormone (FSH) has shown that the ovarian response in terms of number of medium-sized follicles (6–10 mm) and grade A oocytes was better in the FSH than in eCG treatment group (Sendag *et al*., 2008; Ondho *et al*., 2020). In this regard, different studies have been conducted to optimize the dose, route and frequency of administration, and coasting period of FSH treatment in lactating and dry dairy cows (Bó and Mapletoft, 2020). Multiple FSH (200 mg) (da Silva *et al*., 2017) and single FSH (200 mg and 300 mg) (Vieira *et al*., 2016; da Silva *et al*., 2019) regimes were used in Holstein cattle. More recently, to reduce the cost of super-stimulation treatment, an even lower dose of FSH (160 mg), diluted in normal saline or 0.5% hyaluronan, was tested in beef cows. Interestingly, comparable ovarian response and blastocyst production rate were recorded with both low and high dose of FSH (Ongaratto *et al*., 2020).

In a recent study, OPU sessions were conducted in Sahiwal heifers at a random stage of the estrus cycle and the retrieved oocytes were fertilized by using Brahman bull semen (Riaz *et al*., 2021). However, to the best of our knowledge, no comprehensive report on the effect of ovarian stimulation with FSH prior to OPU and fertilization of retrieved oocytes with Sahiwal bull semen is available in Sahiwal cows.

The average body weight (315 kg) of Sahiwal cows is remarkably lower compared to the Holstein Friesian cows (Khan *et al*., 2008), the latter can gain more than 500 kg body weight around second lactation (Archbold *et al*., 2012). Therefore, it was hypothesized that application of a lower dose FSH treatment (100 mg *vs.* 200 mg) will be equally effective in increasing the proportionality of medium-sized follicles, oocyte quality, and blastocyst production rate in Sahiwal cows. Thus, the objectives of the current study were: 1) To monitor the ovarian follicular dynamics following FSH super-stimulation to get appropriate sized follicles for OPU-IVEP, and 2) to evaluate the oocyte quality and *in-vitro* developmental competence of embryos following the ovarian stimulation with two different doses (100 mg and 200 mg) of FSH.

MATERIALS AND METHODS

Animals and management: The current study was executed from February 2020 to April 2021. The off-site Ovum pick-up (OPU) sessions were conducted at the Government Livestock Experimental Station (LES), Bahadarnagar, Okara. Sahiwal cows (n=30), aged 4 to 14 years, with clinically normal reproductive tract, 320±25 kg body weight and 3.25±0.25 BCS, were enrolled. These cows were offered green fodder at the rate of 10% body weight, 2 kg concentrate per cow daily and water *ad libitum*. The experimental animals were handled according to the Guidelines of the Ethical Review Committee, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan.

Study design: Experimental cows were randomly divided into three equal groups (n=10 each). FSH was administered to cows of two groups (FSH-100 mg and FSH-200 mg), while no FSH was given to the third group, which served as control (Vieira *et al*., 2016; da Silva *et al*., 2017; Ongaratto *et al*., 2020). On a random day of the estrus cycle (-48h), all cows underwent ablation of all follicles (≥5 mm) through trans-vaginal ultrasound guided needle puncture (Oliveira *et al*., 2016; Ongaratto *et al*., 2020), and received an intra-vaginal CIDR device (Progesterone 1.9g, Inter Ag, Hamilton, New Zealand). After two days (0h), all cows were treated with 150 µg of D-cloprostenol (Dalmazine®) (da Silva *et al*., 2017; Ongaratto *et al*., 2020). The cows in F100 and F200 groups were injected (i.m.) 100 mg and 200 mg FSH (Folltropin®-V; Vetoquinol, Canada) in four equivalent doses of 25 mg and 50 mg, respectively at 0, 12, 24, and 36 hrs. Ovum pick-up was conducted after 40h of last FSH treatment in FSH-treatment groups and 76 hrs after D-cloprostenol injection in control group, when the CIDR was also removed (Fig. 1). Trans-rectal ultrasonography (Aloka SSD 500, 7.5 MHz frequency) was conducted on daily basis (-24, 0, 24, 48 and 76 hrs) to monitor ovarian follicular dynamics (Fig. 1 and Fig. 2).

Ovum pick-up (OPU): For OPU, the perineal area of the cow was thoroughly washed after administration of epidural anesthesia (5–8 ml of 2% lignocaine). All follicles ≥3 mm were aspirated by using ultrasound guided trans-vaginal 7.5 MHz OPU probe (HCV-4710MV Japan with Honda HS 2200) (Matoba *et al*., 2014). Afterward, the aspirated follicular fluid was filtered through a 70 µm cell strainer (Falcon®) and searched under a stereomicroscope for cumulus-oocyte complexes (COCs). The COCs were categorized into four grades based on morphology and compactness of granulosal layers: Grade I; more than three layers of compact cumulus cells, Grade II; at least a single layer of cumulus cells, Grade III; denuded oocytes, and Grade IV; degenerated cytoplasm, atretic with dark cumulus cells. Grade I, II, and III COCs were considered suitable (viable) for *in-vitro* maturation (da Silva *et al*., 2017; de Carvalho *et al*., 2019).

In-vitro* embryo production: In-vitro* embryo production (IVEP) was carried out at the Embryology Laboratory, Central Laboratory Complex, UVAS, Ravi Campus, Pattoki, Pakistan. All procedures were carried out in IVF-Biosciences media (Bick-land Industrial Park Falmouth, Cornwall, United Kingdom).

All viable COCs (grades I-III) were washed thrice in BO-HEPES-IVM® medium. The viable recovered oocytes of each donor cow were transferred into 2ml cryogenic vial (Corning®) separately containing 1-1.5 ml BO-HEPES-IVM®. The cryogenic vials were kept at 37°C in portable incubator (Minitube) for 24 hrs. After *in-vitro* maturation, COCs were evaluated for cumulus expansion and partially denuded in the BO-Wash®

medium through gentle pipetting. These COCs were further washed thrice in BO-IVF® medium and transferred to 100 µl fertilization drop of BO-IVF®. A straw containing good quality frozen semen of Sahiwal bull (QS-618) was thawed at 37ºC for 30 seconds and semen was mixed with 4ml of BO-Semen Prep®. The supernatant was separated after centrifugation at 392×g for 10 minutes, the sperm pellet was remixed with 4ml BO-Semen Prep® and subjected to centrifugation for 10 minutes. The sperm pellet was evaluated for motility and concentration (Valleh *et al*., 2014; Razza *et al*., 2019). For insemination, each IVF drop received 10–15 µl of semen to attain a final concentration of 2×10^6 live sperm/ml. The COCs and sperm were co-incubated for 16h at 5% CO_2, 38.5ºC temperature, and 95% humidity for IVF. After 16h of insemination, the cumulus cells and excess sperm were removed from presumptive zygotes through gentle pipetting in BO-Wash® medium. After three times washing in BO-IVC® medium, presumptive zygotes from each cow were placed separately in 100 µl drops of BO-IVC® and incubated (Eppendorf-New Brunswick Galaxy 48R) for seven days without changing the culture medium under controlled atmosphere (5% CO_2, 5% O_2 and 90% N_2) at 38.5ºC (Shahzad *et al*., 2020). The cleavage and blastocyst rates were assessed on the third and seventh day of embryo culture.

Embryo transfer and parentage confirmation: One blastocyst stage embryo was loaded in a separate 0.25ml embryo straw by using transfer medium. The loaded straws were transferred from the Embryology Laboratory to LES, Bahadarnagar, Okara in a portable incubator at 37ºC (Stewart *et al*., 2011). Double ovsynch synchronized Sahiwal heifers (n = 9; 2-3 years age) were enrolled as recipients. However, only four heifers at day 7 of estrous cycle were selected for embryo transfer on the basis of quality of corpus luteum (>13 mm). Two blastocyst stage embryos were transferred to each selected heifer. Pregnancy was confirmed at day 30 and 50 by trans-rectal ultrasonography. The parentage of the born calves was confirmed by Triose test (Xin *et al*., 2010; Van de Goor *et al*., 2011) through third-party evaluation at the Quality Control Laboratory, Directorate of Breed Improvement, Livestock and Dairy Development Department, Lahore, Pakistan.

Statistical analysis: The data on number of follicles, retrieved oocytes, cleaved structures, and blastocysts were analyzed by one-way ANOVA, using *SPSS*® Statistics 20. Mean±SEM was used to describe these parameters. Duncan's Multiple Range test was used to compare means among the groups. The proportionality of follicular population among three groups was analyzed by the Chi-Square test.

RESULTS

Follicular population: The data on follicular population in cows of three groups are presented in Table 1. The results revealed that the total number of follicles per OPU session, large-sized (>10mm) follicles and aspirated follicles were significantly (P<0.05) higher in F200 group than the control group; differences between F100 and F200 groups in these parameters were non-significant. Same was true for control and F100 groups. The number of medium sized (6≥10 mm) follicles was significantly (P<0.05) increased in FSH treated (F100 & F200) groups than control, while there was non-significant difference between F100 and. F200 groups. These results suggest that the F100 group may be more economical in terms of yielding the similar number of medium sized follicles when compared with F200 treated group.

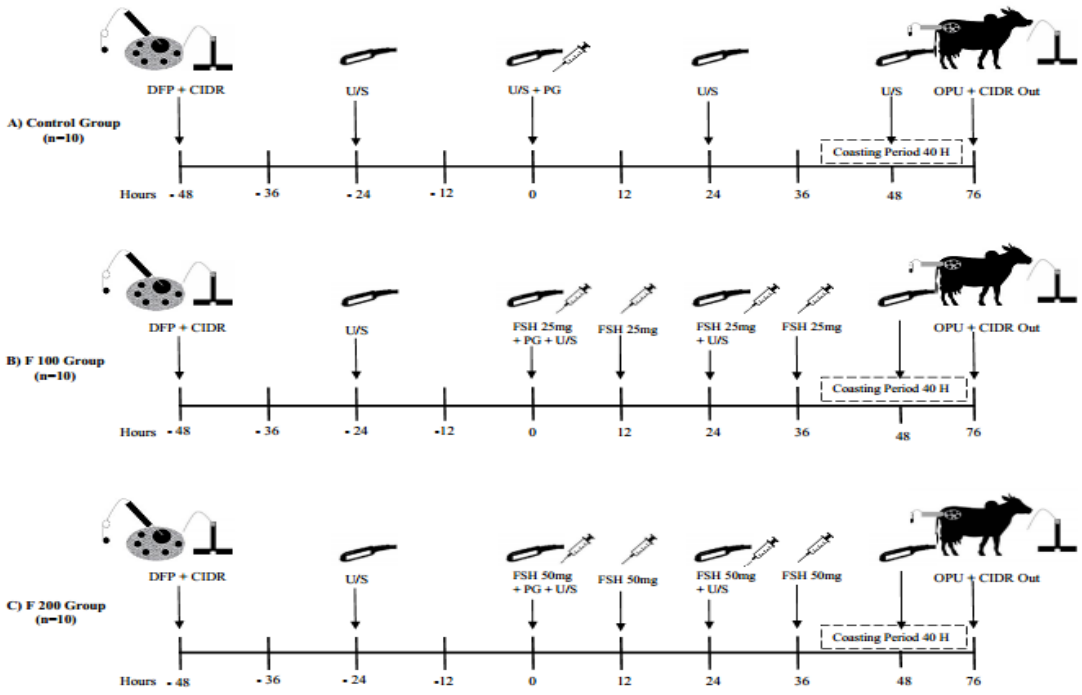

Fig. 1: Schematic representation of the study design to compare the effects of two doses of FSH, i.e., 100 mg (F100) and 200 mg (F200), with the control group in Sahiwal cows. DFP: Dominant follicle puncture; CIDR: Controlled Internal Drug Release; U/S: Ultrasonography; FSH: Follicle stimulating hormone; OPU: Ovum pick-up; PG: Prostaglandin F2α.

Table 1: Effect of two different doses of FSH (100 *vs.* 200 mg) on follicular population and oocytes' number (Mean±SEM) in Sahiwal cows

Parameters	Groups		
	Control	FSH (100 mg)	FSH (200 mg)
Number of OPU sessions	10	10	10
Follicular population			
Total follicles/session	12.4±1.4[a]	16.6±3.4[ab]	20.8±2.8[b]
Small sized follicles (<6mm)	10.9±1.4	6.9±1.8	10.3±1.6
Medium sized follicles (6≥10mm)	1.4±0.3[a]	9.2 ±2.1[b]	9.6±1.6[b]
Large sized follicles (>10mm)	0.1±0.1[a]	0.5±0.2[ab]	0.9±0.3[b]
Aspirated follicles	8.5±1.5[a]	13.9±3.0[ab]	20.0±2.8[b]
Total recovered oocytes	4.8±1.0[a]	6.5±1.7[ab]	9.0±1.2[b]
Total processed oocytes (Grade I to III)	4.0±0.9[a]	6.0±0.8[ab]	7.3±0.9[b]

Different superscripts [(a,b)] represent significance (P≤0.05) within the row.

Follicular dynamics: The ultrasonography-based data on follicular dynamics is depicted in Fig. 2. On analyzing the data, the results affirmed that after 24h (at -24h) of follicular ablation, <3 mm sized follicles were the most dominating structures on ovaries in cows of three groups. At the next 24h (at 0h), no significant change was observed in the follicular growth pattern. Furthermore, at the time of the start of FSH treatments (F100 & F200) (at 0h), the percentage of <3 mm, 3≥6 mm, 6>8 mm and 8≥10 mm sized follicles were statistically similar among all the three groups. Within 24h (at 24h) of first FSH treatment (F100 & F200), the percentage of <3 mm size follicles decreased significantly in FSH treated (F100 & F200) groups as compared to control, which after 48h (at 48h) was minimum for F200 treated group. At the time of OPU (at 76h), the percentage of <3 mm sized follicles remained significantly lower in FSH treated (F100 & F200) groups compared to control (Fig. 2a). The results also revealed that there was statistically no difference in the percentage of 3≥6 mm sized follicles from time of first FSH treatment till 48h between treated and control groups, but a significant decline was noticed in FSH treated (F100 & F200) groups compared to the control at the time of OPU (at 76h) (Fig. 2b).

The percentage of 6>8 mm sized follicles remained significantly higher in both treatment groups than control from 24h of first FSH treatment till the time of OPU. The percentage of 6>8 mm sized follicles was significantly higher in F100 group as compared to the F200 and the control groups (Fig. 2c). These findings indicated a shift of 3≥6 mm sized follicles into a window of 6>8 mm sized follicles under FSH treatment at the time of OPU.

The percentages of 8≥10 mm sized follicles were significantly higher in F200 treated group as compared to control group from 48h of first FSH treatment till the time of OPU. However, both FSH treated groups exhibited a significantly higher percentage of 8≥10 mm sized follicles as compared to control at the time of OPU (at 76h) (Fig. 2d).

Additionally, the proportion of small-sized follicles (>6 mm) recorded at the time of OPU in the control group (87.9%) was significantly higher (P<0.05) compared to F100 (41.57%) and F200 (49.52%) groups. The proportion of medium-sized follicles (6≥10 mm) in the F100 (55.42%) and F200 (46.15%) groups was significantly higher (P<0.05) compared to control group (11.29%). However, large sized follicles (>10 mm) showed non-significant difference among cows of three groups (Fig. 3a).

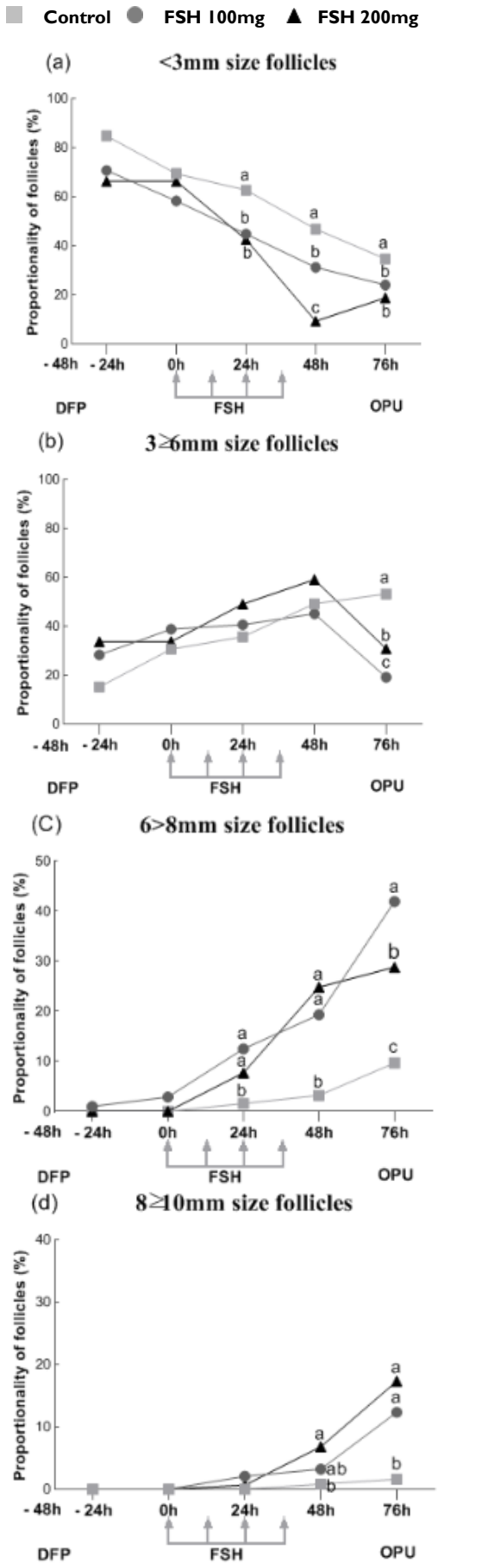

Fig. 2: Follicular dynamics at various intervals post FSH treatments; DFP: Dominant follicle puncture. FSH: Follicle stimulating hormone; OPU: Ovum pick-up; [a-c]denote differences (P≤0.05) among groups at the respective time point.

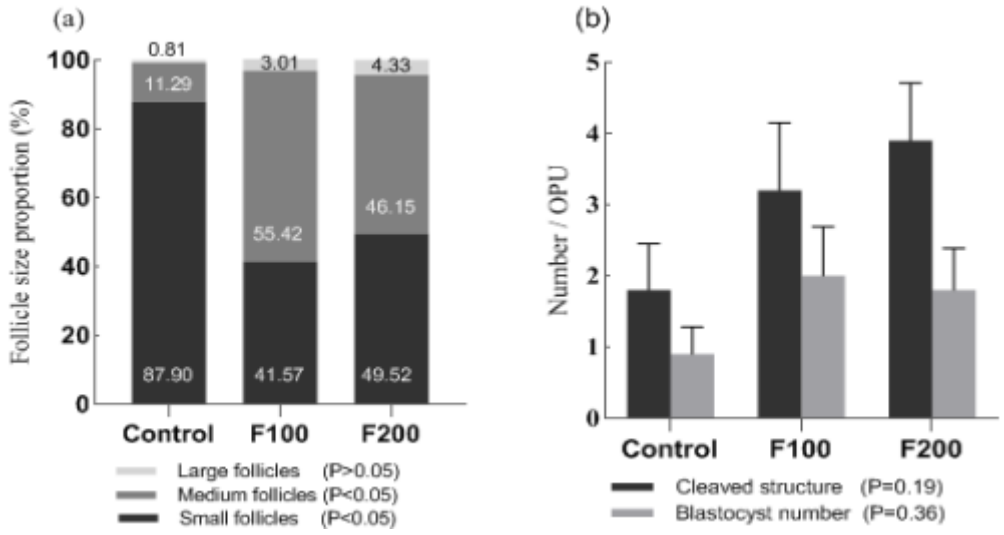

Fig. 3: Effect of FSH (100 *vs.* 200mg) super-stimulation on: (a) Follicular population (small; <6mm, medium; 6-10mm and large; >10mm sized follicles) at the time of OPU in Sahiwal cows, (b) *In-vitro* developmental competence of oocytes, i.e., cleaved structures and blastocysts number per OPU session.

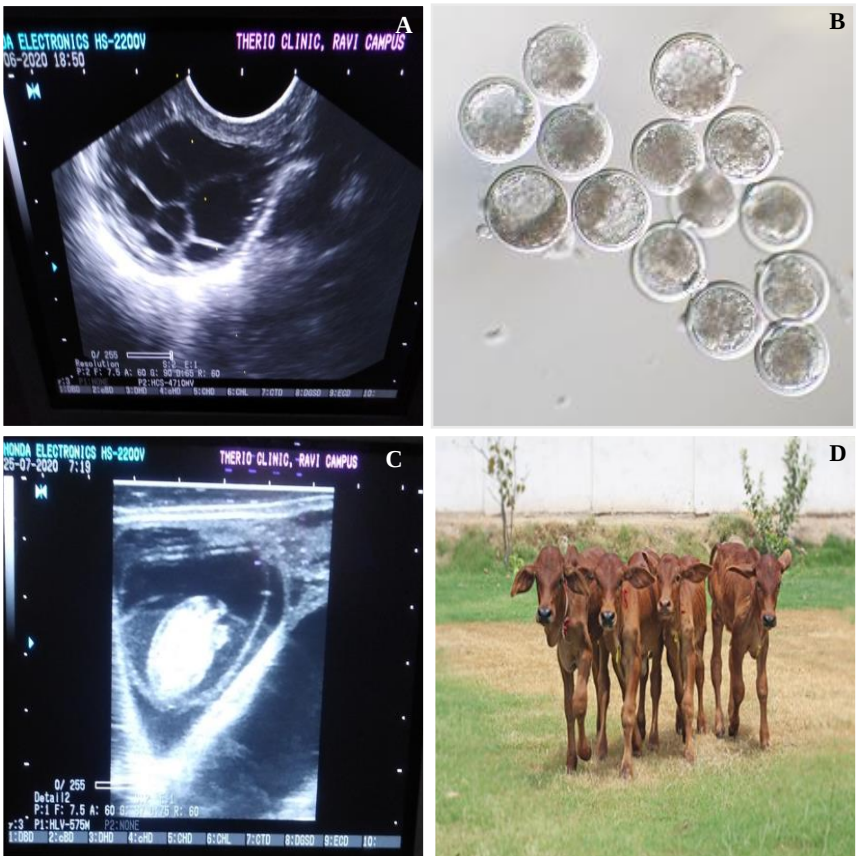

Fig. 4: Ultrasonography of an ovary following FSH stimulation, showing multiple follicles (A); Photograph of some transferable blastocysts produced *in-vitro* (B); Ultrasonography of a pregnant uterus at day 50 (C); Photograph of four Sahiwal calves produced through IVEP (D).

Oocytes' recovery rate and quality: The oocytes' recovery rate was non-significantly different among FSH treated and the control groups (control 56.47%, F100 46.76%, F200 45%). Furthermore, the F200 group yielded significantly (P<0.05) higher number of recovered oocytes and viable oocytes (grade I, II and III) per OPU session than the control group. However, there was no difference in these parameters between control and F100 groups. The same was true for F100 and F200 groups (Table 1).

***In-vitro* developmental competence of oocytes:** The effect of FSH stimulation on *in-vitro* developmental competence of oocytes in Sahiwal cattle is illustrated in Fig. 3b. The data on the number of cleaved structures (control 1.80±0.65, F100 3.2±0.95, F200 3.9±0.8) and blastocysts (control 0.9±0.37, F100 2.0±0.69, F200 1.8±0.59) exhibited non-significant differences among three groups. Nevertheless, the number of blastocysts was numerically two times higher in FSH treated (F100 & F200) groups compared to control, emphasizing the

possibility of increasing sample size that could result in significant results. Similarly, the cleavage rate (48.64, 53.33 and 43.33%, respectively) and blastocyst rate (24.32, 33.33 and 20.00%, respectively) were non-significantly different between control and FSH treated groups.

Pregnancy, calving and parentage confirmation: Amongst four recipient heifers, three were confirmed pregnant, which delivered four healthy calves, including one twin birth. The pregnancy rate of OPU-IVEP embryos was recorded as 75% in Sahiwal heifers. The parentage of OPU-IVEP calves was confirmed after analysis of Electropherograms on Gene mapper version 5.

Ultrasonography of an ovary following FSH stimulation, showing multiple follicles is shown in Fig. 4A, while photograph of some transferable blastocysts produced *in-vitro* is shown in Fig. 4B. Similarly, ultrasonography of a pregnant uterus at day 50 is shown in Fig. 4C, while photograph of four Sahiwal calves produced through IVEP is shown in Fig. 4D.

DISCUSSION

To the best of our knowledge, this is the first study to demonstrate the effect of ovarian stimulation with FSH on follicular dynamics and *in-vitro* developmental competence of oocytes in *Bos indicus* Sahiwal cows. The results depicted that the total number of follicles was significantly higher in the F200 treated group than the control. These results are in close agreement with findings of a previous study, in which enhanced follicular population in beef cows after FSH super-stimulation (control; 8.5±0.6 *vs*. FSH: 14.1±1.0; P<0.05) was reported (Ongaratto *et al*., 2020). Similarly, some recent studies reported an increased number of follicles in a dose-dependent manner in hens (Ghanem and Johnson, 2019), enhanced primordial follicles formation during fetal ovarian development in hamster (Roy and Albee, 2000), and improved primordial follicle reserve in hypogonadal mice (Allan *et al.*, 2006) following FSH treatment, emphasizing the role of FSH in follicular recruitment, in addition to follicular growth. However, Sarwar *et al*. (2020b) reported non-significant difference in number of follicles among FSH treated and the control *Bos taurus* cows (31.43±6.23 and 29.14±6.23, respectively).

In the current study, FSH treatment increased the number and proportionality of medium- sized (6≥10 mm) follicles at the time of OPU due to the transition of small-sized (>6 mm) follicles into medium-sized (6≥10 mm) follicles. Similar findings were made by Vieira *et al*., 2014 (control; 20.8% vs. FSH; 55.1%; P<0.05), Vieira et al., 2016 (control; 19.2% vs. FSH200 64.3%; P<0.05), and da Silva et al., 2017 (control; 10.0% vs. FSH 35.2%; P<0.05). According to Caixeta et al. (2009) and Sarwar et al. (2020a), the oocyte developmental competence is affected by the stage of follicle that highlights the importance of increased proportionality of 6>10 mm sized follicles following FSH treatment.

Our results with respect to aspirated follicles showed significantly higher (P<0.05) values in the F200 group than the control group, which is supported by the findings of a previous study in Holstein cattle by Vieira *et al.* (2016) (control; 16.1±1.1 *vs.* FSH200; 20.4±1.4; P<0.05). The most plausible reason for this increase in aspirated follicles may be the higher proportionality of >6 mm follicles in F200 treated group. In contrast, the number (control; 17.6±1.6 *vs*. FSH 18.2±2.1; P>0.05) noted by Vieira et al. (2014) and percentage of aspirated follicles (control; 82% vs. FSH; 79%; P>0.05) reported by Sarwar *et al*. (2020b) was not affected by FSH super-stimulation of ovaries.

In the present study, the total recovered oocytes were also significantly higher for the F200 treated group compared to the control group. These findings are in accordance with the previous study conducted by Vieira *et al*. (2016) in Holstein cattle (control; 13.1±1.0 *vs*. FSH200; 16.5±1.2; P<0.05). The possible reason may be the higher number of aspirated follicles in F200 group, which in turn resulted in higher recovered oocytes in animals of this group. The results of studies by Vieira *et al*. (2014) (control; 13.0±1.7 *vs*. FSH; 10.7±1.5; P>0.05) and da Silva *et al*. (2017) (control; 19.5±1.6 *vs.* FSH; 18.5±1.27; P>0.05) contradicted our findings.

The number of processed oocytes (Grades I to III) was significantly higher in F200 treated group when compared with the control group, the difference between F100 and F200 groups was non-significant. These findings are strengthened by the results of Vieira *et al*. (2016) (control; 9.3±0.7 *vs.* FSH200; 12.2±1.2; P<0.05) and Ongaratto *et al*. (2020) (contol; 3.7±0.5 *vs.* FSH; 5.5±0.5; P<0.05). This seems to be due to significantly higher ≥6 mm follicles than <6 mm follicles, resulting in increased developmental potential acquired gradually during follicular growth that enhanced total processed oocytes (Sarwar *et al*., 2020a; Zafar *et al*., 2021). Moreover, as the size of the follicle increases and approaches ovulation, the oocyte growing within the follicle becomes more developmentally competent (Sirard *et al*., 2006; Caixeta *et al*., 2009; Sirard, 2011; Sirard, 2012). In contrast, Oliveira *et al*. (2016) (control; 12.3 *vs.* FSH; 10.7; P>0.05) and da Silva *et al*. (2017) (control; 12.6±1.3 *vs.* FSH; 12.7±1.0; P>0.05) reported no improvement in the number of total processed oocytes in FSH super-stimulated groups compared to controls.

The results regarding follicular dynamics revealed gradual transformation of small-sized follicles into medium-sized follicles. At the time of OPU, a higher proportion of 6>8 mm sized follicles was recorded in the F100 treated group. Optimum follicular size (6>8 mm) at the time of OPU was attained under low dose (FSH100), as the dominant follicles >7 mm acquired ovulatory capacity in *Bos indicus* heifers as compared to >10 mm in Holstein cows (Gimenes *et al*., 2008). This follicular transition may be due to activation of c-AMP dependent protein kinase A (Wood and Strauss, 2002) and induction of StAR signaling pathways (Casarini and Crepieux, 2019).

In the present study, the cleaved structures and blastocyst number were almost two times higher in FSH treated (F100 and F200) groups than the control group, which is in agreement with the previous studies in which embryos produced per OPU reported by Vieira *et al*. (2016) (control; 2.4±0.5 *vs.* FSH200; 3.7±0.7; P<0.05) and transferable embryos recorded by Sarwar *et al*. (2020b) (control; 3.33±0.75 *vs.* FSH; 4.10±0.75; P<0.05)

were significantly higher in FSH treated groups. The increased blastocyst yield may probably be due to higher total follicular number and aspirated follicles in FSH treated groups than the control group. In contrast, our findings in terms of blastocyst yield are not supported by the results documented by Oliveira *et al*. (2016) (control; 2.6 *vs*. FSH; 3.0; P>0.05) and da Silva *et al*. (2017) (control; 4.1±0.5 *vs*. FSH; 4.3±0.5; P>0.05), who recorded no difference in blastocyst yield between FSH treated and control groups. This might have been due to non-significant difference in total number of follicles and recovered oocytes between FSH treated and control groups in these studies.

The current study is the first one to document birth of four pure Sahiwal calves in Pakistan through OPU-IVEP, following fertilization of oocytes retrieved from FSH stimulated Sahiwal cows with semen from a Sahiwal bull. Different synchronization protocols have been used previously for increasing pregnancies in bovine recipients (Bo' *et al*., 2012). In the present study, double Ovsynch synchronization protocol resulted in 75% (3/4) pregnancy rate in recipient Sahiwal heifers which are comparable with previous reports where progesterone, GnRH and estradiol based synchronization protocols were used to synchronize the recipient cows prior to embryo transfer (Nasser *et al*., 2004; Bonacker *et al*., 2020).

Conclusions: The results of this study established the positive ovarian response to FSH (200 mg) stimulation in Sahiwal cows. Furthermore, the FSH stimulation significantly improved proportionality of medium-sized follicles. The number of suitable follicles for aspiration was significantly increased in F200 than the control group. Besides, low dose of FSH (100 mg) was numerically (P>0.05) equally efficient as high dose (200 mg) in blastocyst yield per OPU session, highlighting the significance of economic preference in the OPU-IVEP program, particularly in Sahiwal cows with relatively lower average body weight compared to *Bos taurus* cows.

Authors contribution: MN and AR conceived the idea and finalized experimental design. MN, MS, MY and MS performed OPU-IVEP and embryo transfer. MN and MS executed data curation, statistical analysis and writing of original draft. MS, IZ, AR and NA critically reviewed the manuscript. There is no conflict of interest among authors.

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REFERENCES

Allan CM, Wang Y, Jimenez M, *et al*., 2006. Follicle-stimulating hormone increases primordial follicle reserve in mature female hypogonadal mice. J Endocrinol 188:549-57.

Archbold H, Shalloo L, Kennedy E, *et al*., 2012. Influence of age, body weight and body condition score before mating start date on the pubertal rate of maiden Holstein–Friesian heifers and implications for subsequent cow performance and profitability. Animal 6:1143-51.

Bo' G, Baruselli PS and Mapletoft RJ, 2012. Increasing pregnancies following sychronization of bovine recipients. Anim Reprod 9:312-7.

Bó GA and Mapletoft RJ, 2020. Superstimulation of ovarian follicles in cattle: gonadotropin treatment protocols and FSH profiles. Theriogenology 150:353-9.

Bonacker RC, Gray KR, Breiner CA, *et al*., 2020. Comarison of the 7 & 7 synch protocol and the 7-day CO-Synch+CIDR protocol among receipent beef cows in an embryo tranfer program. Theriogenology 158:490-6.

Caixeta ES, Ripamonte P, Franco MM, *et al*., 2009. Effect of follicle size on mRNA expression in cumulus cells and oocytes of *Bos indicus*: an approach to identify marker genes for developmental competence. Reprod Fertil Dev 21:655-64.

Casarini L and Crépieux P, 2019. Molecular mechanisms of action of FSH. Front Endocrinol 10:305. doi: 10.3389/fendo.2019.00305.

da Silva BLM, Adona PR, Guemra S, *et al*., 2019. Ovum pick up: cows treated with single doses of follicle stimulating hormone. J Agri Sci 11:231-9.

da Silva JCB, Ferreira RM, Filho MM, *et al*., 2017. Use of FSH in two different regimens for ovarian superstimulation prior to ovum pick up and *in vitro* embryo production in Holstein cows. Theriogenology 90:65-73.

de Carvalho JGS, de Carvalho NAT, Bayeux BM, *et al*., 2019. Superstimulation prior to the ovum pick-up improves the *in vitro* embryo production in nulliparous, primiparous and multiparous buffalo (*Bubalus bubalis)* donors. Theriogenology 138:164-8.

Dieleman SJ, Hendriksen PJM, Viuff D, *et al*., 2002. Effects of *in vivo* prematuration and *in vivo* final maturation on developmental capacity and quality of pre-implantation embryos. Theriogenology 57:5-20.

Gandolfi TBA and Gandolfi F, 2001. The maternal legacy to the embryo: cytoplasmic components and their effects on early development. Theriogenology 55:1255-76.

Gimenes LU, Sá Filho MF, Carvalho NAT, *et al*., 2008. Follicle deviation and ovulatory capacity in *Bos indicus* heifers. Theriogenology 69:852-8.

Ghanem K and Johnson AL, 2019. Response of hen pre-recruitment ovarian follicles to follicle stimulating hormone *in vivo*. Gen Comp Endocrinol 270:41-7.

Khan MS, Rehman Z, Khan MA, *et al*., 2008. Genetic resources and diversity in Pakistani cattle. Pak Vet J 28:95-102.

Lonergan P, Monaghan P, Rizos D, *et al*., 1994. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture *in vitro*. Mol Reprod Dev 37:48-53.

Matoba S, Yoshioka H, Matsuda H, *et al*., 2014. Optimizing production of *in vivo*-matured oocytes from superstimulated Holstein cows for *in vitro* production of embryos using X-sorted sperm. J Dairy Sci 97:743-53.

Moore SG and Hasler JF, 2017. A 100-Year Review: Reproductive technologies in dairy science. J Dairy Sci 100:10314-31.

Nasser LF, Reis EL, Oliveria MA, *et al*., 2004. Comparison of four sychronization protocols for fixed-time boine embryo transfer in *Bos indicus* x *Bos taurus* recipients. Theriogenology 62:1577-84.

Oliveira LH, Sanches CP, Seddon AS, *et al*., 2016. Follicle superstimulation before ovum pick-up for *in vitro* embryo production in Holstein cows. J Dairy Sci 99:9307-12.

Ondho YS, Samsudewa D and Lestari DA, 2020. *In vitro* maturation of ovine oocytes using follicle stimulating hormone (FSH), estradiol-17β and co-culture of fallopian tube epithelial cells (FTEC) in tissue culture medium-199 (TCM-199). Int J Vet Sci 9:66-71.

Ongaratto FL, Cedeño AV, Rodriguez-Villamil P, *et al*., 2020. Effect of FSH treatment on cumulus oocyte complex recovery by ovum pick up and *in vitro* embryo production in beef donor cows. Anim Reprod Sci 214: 106274. doi.org/10.1016/j.anireprosci.2020. 106274.

Razza EM, Pedersen HS, Stroebech L, *et al*., 2019. Simulated physiological oocyte maturation has side effects on bovine oocytes and embryos. J Assist Reprod Genet 36:413-24.

Riaz H, Farooq MO and Farooq M, 2021. Birth of first IVF calves in Sahiwal cows of Pakistani origin. Pak Vet J 41:303-5.

Roy SK and Albee L, 2000. Requirement for follicle-stimulating hormone action in the formation of primordial follicles during perinatal ovarian development in the hamster. Endocrinology 141:4449-56.

Sarwar Z, Saad M, Saleem M, *et al*., 2020a. Effect of follicle size on oocytes recovery rate, quality, and *in-vitro* developmental competence in *Bos indicus* cows. Anim Reprod 17: e20200011; doi.org/10.1590/1984-3143-AR2020-0011

Sarwar Z, Sagheer M, Sosa F, *et al*., 2020b. Meta-analysis to determine effects of treatment with FSH when there is progestin-priming on *in-vitro* embryo production using ovum pick-up in *Bos taurus* cows. Anim Reprod Sci 221: 106590. doi.org/10.1016/j.anireprosci. 2020.106590

Sendag S, Cetin Y, Alan M, *et al*., 2008. Effects of eCG and FSH on ovarian response, recovery rate and number and quality of oocytes obtained by ovum pick-up in Holstein cows. Anim Reprod Sci 106:208-14.

Seneda MM, Zangirolamo AF, Bergamo LZ, *et al*., 2020. Follicular wave synchronization prior to ovum pick-up. Theriogenology 150:180-5.

Singh J, Dominguez M, Jaiswal R, *et al*., 2004. A simple ultrasound test to predict the superstimulatory response in cattle. Theriogenology 62:227-43.

Sirard MA, 2011. Follicle environment and quality of *in vitro* matured oocytes. J Assist Reprod Genet 28:483-8.

Sirard MA, Richard F, Blondin P, *et al*., 2006. Contribution of the oocyte to embryo quality. Theriogenology 65:126-36.

Sirard MA, 2012. Factors affecting oocyte and embryo transcriptomes. Reprod Domest Anim 47(Suppl 4):148-55.

Shahzad Q, Pu L, Wadood AA, *et al*., 2020. Proteomics analysis reveals that warburg effect along with modification in lipid metabolism improves *in vitro* embryo development under low oxygen. Int J Mol Sci 21: 1996. doi.org/10.3390/ijms21061996.

Stewart BM, Block J, Morelli P, *et al*., 2011. Efficacy of embryo transfer in lactating dairy cows during summer using fresh or vitrified embryos produced *in vitro* with sex-sorted semen. J Dairy Sci 94:3437-45.

Valleh MV, Hyttel P, Rasmussen MA, *et al*., 2014. Insulin-like growth factor 2: a modulator of anti-apoptosis related genes (HSP70, BCL2-L1) in bovine preimplantation embryos. Theriogenology 82:942-50.

Van de Goor LHP, Koskinen MT and van Haeringen WA, 2011. Population studies of 16 bovines STR loci for forensic purposes. Int J Legal Med 125:111-19.

Viana JHM, 2018. 2017 Statistics of embryo production and transfer in domestic farm animals. Embryo Technology Newsletter 36:8-25.

Vieira LM, Rodrigues CA, Netto AC, *et al*., 2016. Efficacy of a single intramuscular injection of porcine FSH in hyaluronan prior to ovum pick-up in Holstein cattle. Theriogenology 85:877-86.

Vieira LM, Rodrigues CA, Netto AC, *et al*., 2014. Superstimulation prior to the ovum pick-up to improve *in vitro* embryo production in lactating and non-lactating Holstein cows. Theriogenology 82:318-24.

Wood JR and Strauss JF, 2002. Multiple signal transduction pathways regulate ovarian steroidogenesis. Rev Endocr Metab Disord 3:33-46.

Xin Y, Zan L, Liu Y, *et al*., 2010. Population genetic analysis of 6 Y-STR loci in Chinese northwestern Qinchuan yellow cattle breed. Mol Biol Rep 37:3043-9.

Zafar MA, Ali F, Lodhi LA, *et al*., 2021. Effect of tocopherol and selenium on the performance of buserelin for estrus induction in late season anestrus mares (Equus caballus). Agrobiol Rec 4:8-13.