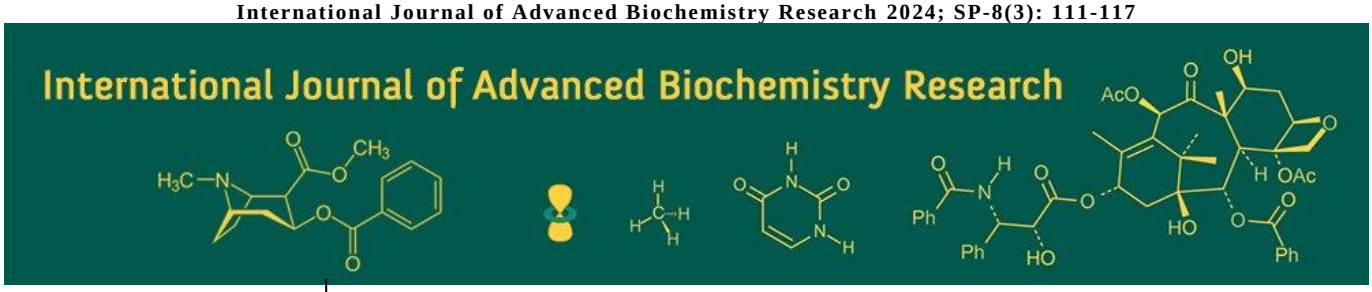

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; SP-8(3): 111-117
www.biochemjournal.com
Received: 13-12-2023
Accepted: 23-01-2024

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Effect of follicle stimulating hormone stimulation on *in-vitro* oocyte maturation in Sahiwal cattle

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DOI: https://doi.org/10.33545/26174693.2024.v8.i3Sb.702

Abstract
The current investigation took place at the College of Veterinary Science, Korutla, Telangana, within the framework of the Embryo Transfer & *In-Vitro* Fertilization (ET & IVF) project. The objective was to assess the impact of Follicle Stimulating Hormone (FSH) stimulation on ovarian follicular growth, oocyte yield, oocyte quality, and *In-Vitro* maturation (IVM) of oocytes. In this study, irrespective of the estrous cycle, 20 non-lactating Sahiwal cows were evenly divided into two groups. Group 1 animals (non-stimulated, n = 10) underwent ovum pick-up (OPU) once at a random stage of the estrous cycle. In Group 2 animals (FSH-Stimulated, n = 10), at a random stage of the estrous cycle (Day 0), a progesterone (P4)-releasing intravaginal device (CIDR) was inserted, and from day 4 onwards, they were treated with injection FSH (200mg) intramuscularly in 8 tapering doses with 12-hour intervals. After a 36-hour coasting period, CIDR was removed, and oocytes were recovered by OPU. The retrieved oocytes were incubated for 24 hours for *in-vitro* Maturation. After 24 hours of IVM, the oocytes were assessed for maturation. Follicle Stimulating Hormone treatment ($p<0.05$) resulted in an increase in the total number of follicles (non-stimulated, 7.30 ± 0.36 vs. FSH-stimulated, 21.50 ± 1.67), the number of medium follicles (4 - 8 mm; non-stimulated, 2.00 ± 0.25 vs. FSH-treated, 11.4 ± 1.04), large follicles (non-stimulated, 0.7 ± 0.15 vs. FSH-stimulated, 7.90 ± 1.15), total number of oocytes recovered per animal (non-stimulated, 6.10 ± 0.27 vs FSH-stimulated, 13.70 ± 1.19), and an increase in the number of good quality oocytes. The study concluded that FSH stimulation was effective in stimulating the growth of the follicular population prior to OPU and enhancing *In-Vitro* oocyte competence for *in-vitro* embryo production in non-lactating Sahiwal cows.

Keywords: Follicle, stimulating, stimulation, oocyte, maturation

Introduction
Assisted reproductive technologies (ART) play a crucial role in enhancing reproductive efficiency and bolstering the population of genetically superior cattle (Sirard, 2018) [20]. Artificial insemination effectively disseminates the genetics of high-yielding sires, while the incorporation of embryo transfer (ET) technologies with Ovum Pick Up (OPU) provides additional benefits in terms of propagating dam genetics (Wrenzycki, 2018) [25] and also a promising alternative for preserving breeds and accelerating the multiplication of superior germplasm (Baldassarerre *et al*., 2021) [2].

Increasing the number of viable embryos is a key factor in enhancing reproductive outcome. One notable approach to increase viable embryos include involves follicle-stimulating hormone (FSH) pre-treatment before ovum pick-up, recognized as a straightforward, cost-effective, and promising strategy to improve oocyte retrieval efficiency (Hayden *et al*., 2022) [12]. Superstimulation protocols employing FSH have demonstrated the ability to boost the population of developmentally competent oocytes, thereby benefiting OPU-IVEP programs (Silva *et al*., 2017) [6].

The success of IVEP is significantly dependent on ovarian follicle development, as well as the quality and quantity of retrieved oocytes. Oocytes derived from bovine ovary follicles with a diameter of 3 mm undergo RNA synthesis and exhibit substantial growth, acquiring the capacity to form blastocysts post-fertilization (Blondin & Sirard, 1995; Fair *et al*., 1995; Hyttel *et al*., 1997) [3, 10, 13].

Consequently, in Ovum Pick-Up/*In-Vitro* Embryo Production (OPU/IVEP) programs, the majority of harvested oocytes are obtained from follicles with diameters ranging between 3 and 8 mm, occurring without a preovulatory LH surge, highlighting the importance of the follicular microenvironment before ovulation (Dieleman *et al*., 2002) [7].

Currently, OPU and IVEP are widely employed in B. taurus breeds globally. While the initial work on OPU in Indian cattle was described by Manik *et al*. (2003) [17], information regarding the impact of hormonal pre-treatment on the number of follicles available for puncture, oocyte recovery rate, oocyte quality, and blastocyst development rate in Sahiwal cows is limited. Therefore, the aim of this study is to investigate the effects of FSH pre-stimulation on follicle number and size, oocyte competence, and subsequent *In-Vitro* embryo production in Sahiwal cows.

Materıals and Methods

Experimental location and experimental animals

The current investigation was carried out within the framework of the Embryo Transfer & *In-Vitro* Fertilization (ET & IVF) project at the Department of Veterinary Gynaecology & Obstetrics, College of Veterinary Science, Korutla, Jagitial district, Telangana, spanning from January to October 2023. A cohort of twenty Sahiwal cows (Bos indicus), aged 3-6 years and weighing between 250 to 450 kg, were selected as oocyte donors and randomly allocated into two groups. The cows received optimal nutritional care, had access to ad libitum water, and underwent regular health assessments, deworming, and vaccinations against infectious diseases to ensure their well-being.

Experimental design

Irrespective of the phase in the estrous cycle, twenty cows were equitably divided into two cohorts. Animals in Group 1 (non-stimulated, n = 10) underwent a single ovum pick-up (OPU) at a randomly stage of the estrous cycle. On the other hand, cows in Group 2 (FSH Stimulated, n = 10) were administered an intravaginal progesterone device (progesterone: 1.38 gm, Eazi Breed CIDR) on day 0, at a randomly determined stage of the estrous cycle, and were subjected to stimulation with follicle-stimulating hormone [Inj. Follitropin V (Vetoquinol, Canada, containing pituitary extract of porcine follicle-stimulating hormone (pFSH) 400mg NIH)] at a dosage of 200mg, administered in 8 tapering doses at 12-hour intervals intramuscularly, commencing from day 4 of CIDR application.

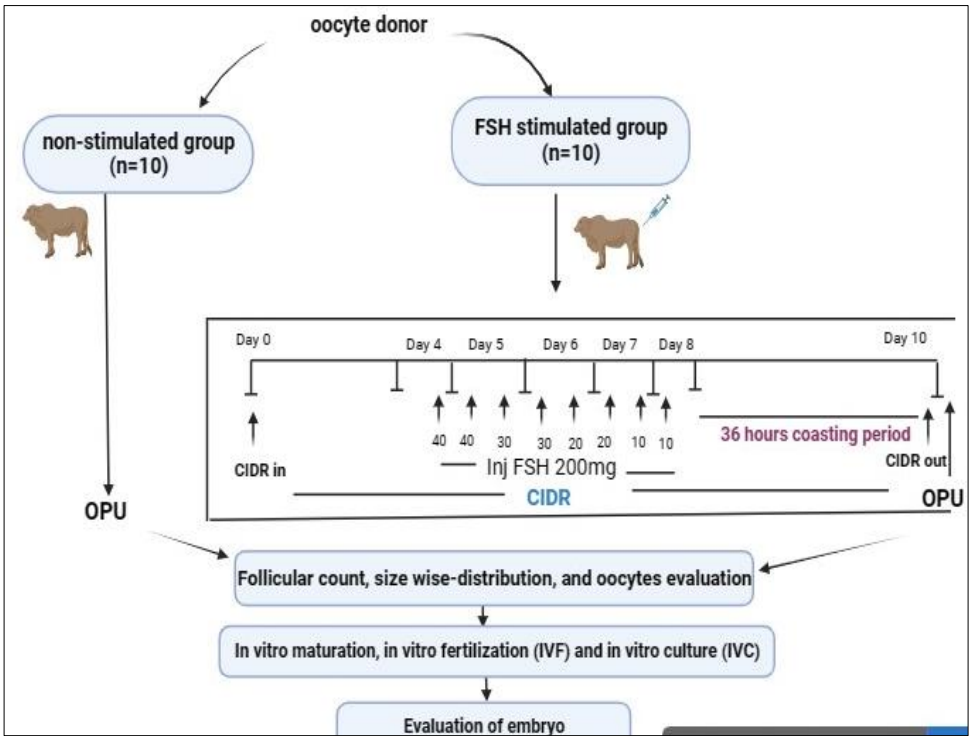

Fig 1: Experimental Design

During the Ovum Pick-Up (OPU) procedure, the animal was restrained in a squeeze chute, and rectal clearance was executed through back raking. Caudal epidural anesthesia (3-5 ml of 2% Lignocaine Hydrochloride) was administered to prevent defecation and abdominal straining, facilitating the manipulation of the ovaries. The vulva and perineal area were cleansed with plain water, and the tail was secured to one side with a cotton rope. Prior to the insertion of the vaginal probe, the vulval lips underwent swabbing with tissue paper soaked in 70% alcohol.

During the Ovum Pick-Up (OPU) procedure, the ovaries underwent manipulation per rectum, with either the right or left ovary positioned between the fingers. Following meticulous cleansing, a lubricated transvaginal probe with a glove was introduced into the anterior vagina and positioned to the left or right side of the cervix. The ovary was gently manipulated against the probe head to achieve a clear visualization of follicles on the monitor. The number and diameter of follicles were recorded and classified as small (<4mm), medium (4-8 mm), or large (≥8 mm) based on their dimensions.

Furthermore, the presence of the corpus luteum and the diameter of the corpus luteum were duly documented. After achieving stabilization of the ovary and the targeted follicle, a needle equipped with an aspiration line was introduced through the plastic probe carrier into the follicle antrum.

Follicular fluid was subsequently aspirated utilizing a continuous negative pressure of 90mmHg. The needle was rotated to curette the follicle and dislodge the oocyte if necessary. Prior to aspirating the next follicle, the needle was withdrawn and rinsed with pre-heated OPU recovery media to prevent clotting or oocyte adherence. The oocyte recovery rate was calculated as the percentage of oocytes retrieved from aspirated follicles for each cow. Successful aspirations were confirmed by the disappearance of follicle images on-screen. This process was reiterated for both ovaries of each cow, utilizing a separate needle for each donor.

Oocyte recovery

Following the aspiration of each ovary, the 50ml centrifuge tube housing the follicular aspirate was promptly transported to the laboratory.In the oocyte collection room, the tube was positioned in a pre-warmed dry bath. The contents aspirated into the tube underwent filtration through a 75µ oocyte mini filter. The centrifuge tube underwent 3-4 rinses with OPU media, and the filtered contents were discharged into a 100mm petri dish. The filter was washed with 20 ml OPU media within the same petri dish and subsequently examined under a stereozoom microscope at 1x magnification to discern cumulus-oocyte complexes (COCs). The identified COCs were then transferred to WASH media (Vitrogen, Brazil), underwent a washing process, and were examined under a stereozoom microscope at 8x magnification for the purpose of grading.

Assessment of Cumulus-Oocyte Complexes (COCs)

In this investigation, the classification of Cumulus-Oocyte Complexes (COCs) into grades 1 to 4 was executed, based on quantity and organization of cumulus cell layers and the homogeneity of ooplasm, as elucidated by Goodhand *et al.* (2000) and Manik *et al.* (2003) [17].

***In-vitro* maturation of oocytes**

For *in vitro* maturation (IVM), the *in vitro* maturation media (IVM) (Vitrogen, Brazil) underwent equilibration in a CO2 incubator (5% CO2 in air, 38.5 °C temperature, and humidity exceeding 90%) overnight to stabilize the pH before the IVM procedure. The cumulus-oocyte complexes (COCs), having been washed 2-3 times in wash media, were subsequently rinsed twice with IVM media and then subjected to *in vitro* maturation. For IVM, group of around 20 COCs were placed in equilibrated 500µl of IVM media, overlaid with 300µl sterile mineral oil (Vitrogen, Brazil) in 5 well dish (Minitube, Germany) and kept for maturation in a humidified CO_2 incubator (5% CO_2 in air and more than 90% RH) at 38.5 °C for 24 hrs. After a 24-hour period of *in vitro* maturation, the maturation status of the oocytes was assessed, taking into consideration the degree of expansion of the cumulus cell mass and the extrusion of the first polar body into the perivitelline space.

Statistical analysis

The collected data underwent statistical analysis employing descriptive statistics, and the significance of means was assessed through Tukey's HSD test utilizing SPSS (2009) version 16.

Results

Twenty sessions of OPU were conducted, comprising ten sessions each for non-stimulated and FSH-stimulated Sahiwal cows. The information concerning the follicle count and the size distribution of follicles in Sahiwal cows undergoing transvaginal ovum pick-up is outlined in Table 1. The average number of follicles eligible for aspiration, the mean count of medium-sized follicles, and the mean count of large follicles demonstrated a statistically significant increase (p<0.05) in the FSH-stimulated group compared to the non-stimulated group.

Table 1: Mean follicular population available for aspiration and mean follicle size distribution

Follicular size distribution	Mean number of follicles (Non-stimulated) (mean ± SEM)	Mean number of follicles (FSH stimulated) (mean ± SEM)
Small (<4mm)	4.60 ± 0.30	2.90 ± 0.27
Medium (4-8mm)	2.00 ± 0.25	11.7 ± 1.04
Large (>8mm)	0.7 ± 0.15	6.90 ± 0.56
Total follicular population	7.30 ± 0.36	21.50 ± 1.67

Values within a row differ significantly (p<0.05) in between FSH stimulated and non-stimulated group.

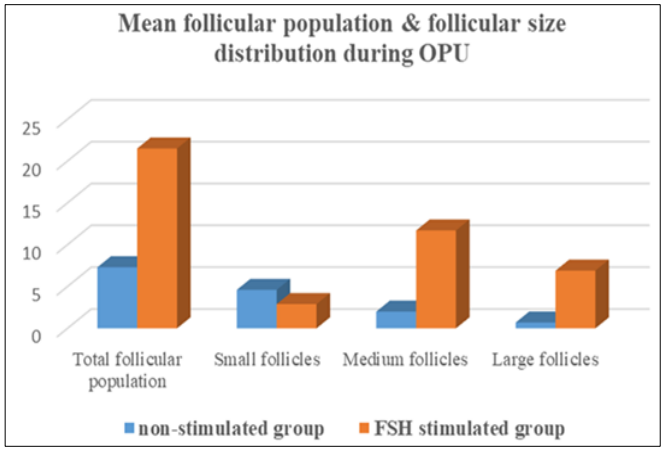

Fig 2: Mean follicular population eligible for aspiration and the distribution of mean follicle sizes.

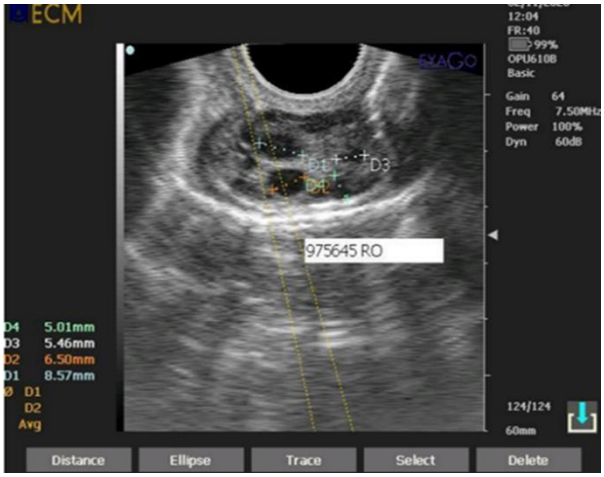

Fig 3: Ultrasonographic image of non-stimulated ovary

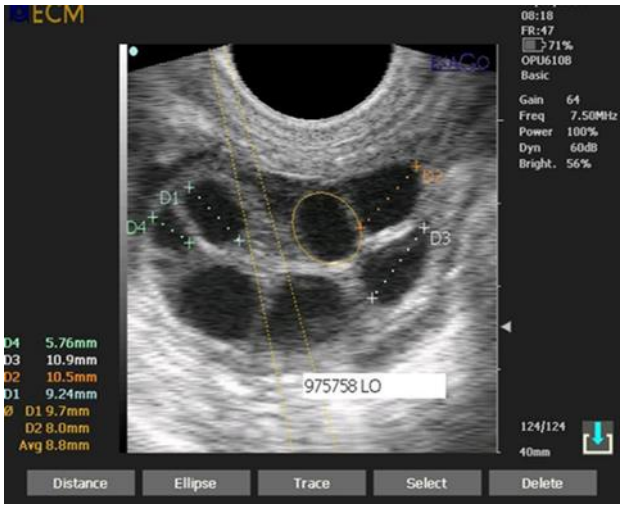

Fig 4: Ultrasonographic image of FSH stimulated ovary

The results of the investigation pertaining to the oocyte retrieval rate, mean number of oocytes recovered per animal, and the distribution of oocytes across grades 1, 2, 3, and 4 are illustrated in Table 2 and Figure 3. The mean total count of recovered oocytes and the mean number of grade 1 oocytes acquired per animal were markedly higher in the FSH-stimulated group compared to the non-stimulated group, with statistical significance (p<0.05).

Table 2: Mean oocyte yield and mean oocyte quality per cow in non-stimulated and FSH stimulated cows

	Average count of oocytes of various grades retrieved	
Attribute	**Non-stimulated group (mean ± SEM)**	**FSH stimulated group (mean ± SEM)**
Grade 1	1.30 ± 0.42	4.80 ± 0.64*
Grade 2	2.50 ± 0.42	4.10 ± 0.80
Grade 3	2.00 ± 0.55	4.00 ± 0.80
Grade 4	0.30 ± 0.15	0.40 ± 0.16
Total oocytes recovered	6.1 ± 0.27	13.30 ± 1.19*

*values with in row differ significantly

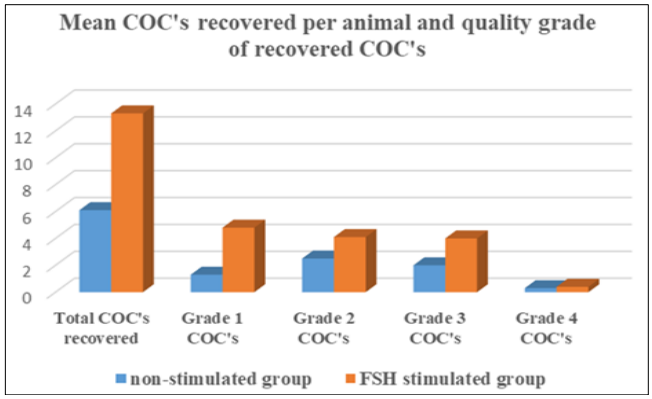

Fig 5: Mean number of Cumulus Oocyte Complexes recovered per animal

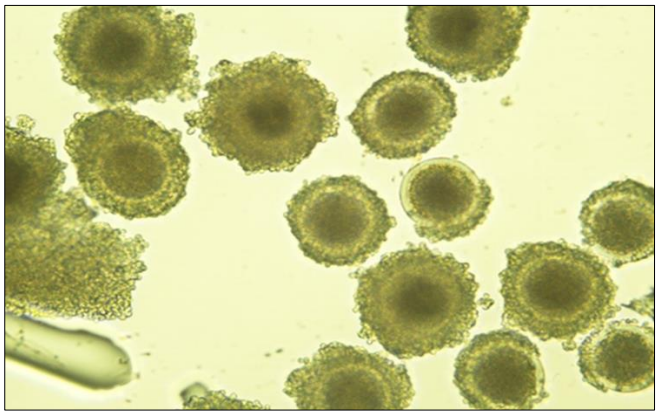

Fig 6: Immature oocytes retrieved through Ovum Pick Up

The capacity of immature oocytes obtained through Ovum Pick-Up (OPU) for rates of cumulus cell expansion and 1st polar body extrusion during *in vitro* maturation reveals no statistically significant distinction (p<0.05) between the FSH-stimulated and non-stimulated groups (refer to Table 3). Nevertheless, the average number of oocytes exhibiting cumulus cell expansion and 1st polar body extrusion is significantly greater in the FSH-stimulated group in comparison to the non-stimulated group (refer to Table 3).

Table 3: Effect of FSH stimulation on expansion of cumulus cells and extrusion of 1st polar body into per vitelline space.

	Non-stimulated group	FSH stimulated group
Mean number of COC's showing cumulus cell expansion per animal	5.40 ±0.22	11.70 ± 1.07*
Mean cumulus cell expansion rate (%) (mean ± SEM)	93.32 ± 2.72	91.01 ± 1.79
Mean number of COC's showing 1st polar body extrusion in to perivitelline space of oocyte per animal	3.50 ± 0.16	8.40 ± 0.74*
First polar body extrusion rate (%) (mean ± SEM)	60.35 ± 2.05	65.53 ± 1.86

*values with in row differ significantly

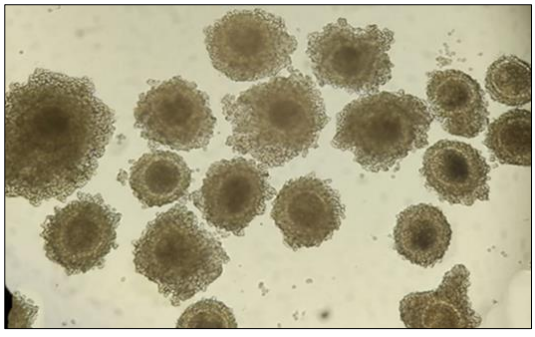

Fig 7: Immature oocytes retrieved through Ovum Pick Up before IVM (A- grade 1 oocyte, B- grade 2 oocytes, C- grade 3 oocytes (under 10× of phase contrast microscope)

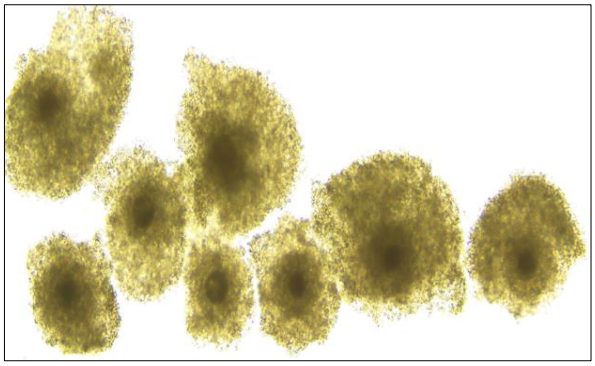

Fig 8: Cumulus Oocyte Complexes showing expansion of cumulus cells after IVM (under 10× of phase contrast microscope)

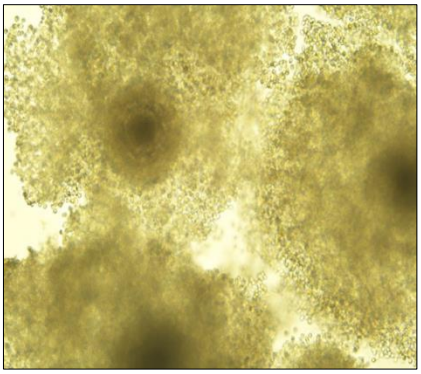

Fig 9: Expanded cumulus cells after 24hrs of IVM (under 20× of phase contrast microscope)

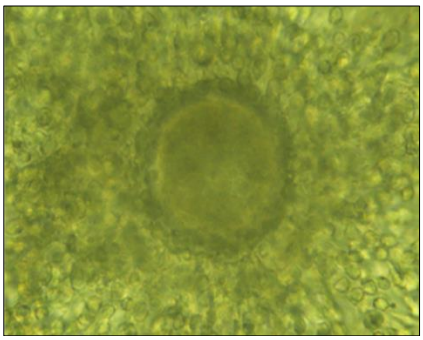

Fig 10: Expanded cumulus cells after 24hrs of IVM (under 40× of phase contrast microscope)

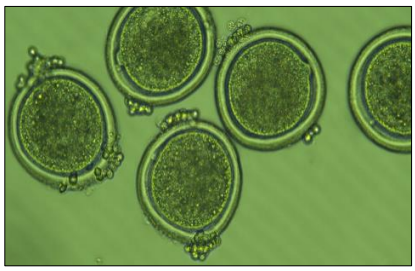

Fig 11: Oocytes showing first polar body extrusion in to perivitelline space after 24hrs of IVM (under 20× of phase contrast microscope)

Discussion

The primary objective of this present investigation was to assess the impact of ovarian stimulation on the yield, quality, and maturation rates in *in-vitro* of Sahiwal oocytes. In this investigation, the mean quantity of follicles eligible for aspiration within the FSH-stimulated cohort (21.50 ± 1.67) exhibited a statistically significant elevation (p<0.05) compared to the non-stimulated group (7.30 ± 0.36) which indicates that FSH administration was crucial in promoting follicle growth, protecting subordinate follicles from atresia, ensuring continuous growth, and ultimately increasing the pool of follicles available for aspiration. According to earlier studies (Vieira *et al*., 2016) [24], these results are similar. In contrast, some studies reported no discernible impact of exogenous FSH on total follicle numbers during Ovum Pick-Up (OPU) (Vieira *et al*., 2014; Silva *et al*., 2017; Egashira *et al*., 2019; Ongaratto *et al*., 2020; Vennapureddy *et al*., 2022; Cameron *et al*., 2023; Krishna *et al*., 2023) [24, 6, 9, 5, 15, 18, 22, 5, 15]. Variations in follicular availability among FSH-treated cows in diverse studies may be attributed to extrinsic factors such as breed, age, cyclicity, and nutritional status. Additionally, intrinsic factors including the reserve pool's follicle count, follicular recruitment, development, and atresia could contribute to this variability.

Follicle size distribution in the present study was markedly influenced by FSH stimulation. These results corroborate the notion that exogenous FSH administration stimulates follicle growth, rescuing subordinate follicles from atresia, facilitating continuous growth, and acquisition of a dominant phenotype.

The present investigation reveals a noteworthy increase (p<0.05) in the mean count of retrieved oocytes per individual following stimulation with Follicle-Stimulating Hormone (FSH) (FSH-stimulated group: 3.30 ± 1.19) compared to the non-stimulated group (6.10 ± 0.27). However, the recovery rate is lower in the FSH-stimulated group (63.46 ± 4.98%) than in the non-stimulated group (83.78 ± 2.3%). These results align with existing literature, including studies by Jeyakumar (2004) [14], Aller *et al*. (2012) [1], Silva *et al*. (2017) [6], Srimannarayana (2019) [21], Ongaratto *et al*. (2020) [18], highlighting a higher percentage of recovered oocytes in non-stimulated groups. The optimal oocyte retrieval efficacy from small follicles, ascribed to diminished intra-follicular pressure, thereby mitigating losses during the process of Ovum Pick-Up (OPU). The low oocyte retrieval efficiency observed in the follicle-stimulating hormone (FSH) induced cohort in this investigation could be ascribed to the heightened prevalence of medium and large follicles within the FSH-stimulated group. This cohort encompasses a higher viscosity of follicular fluid, potentially resulting in more mature oocytes, and the aspirate may predominantly consist of layers of granulosa cells. In this investigation, the average number of retrieved oocytes per individual exhibits a statistically significant increase (p<0.05) in the FSH-stimulated group (13.30 ± 1.19) compared to the non-stimulated group (6.10 ± 0.27). These findings diverge from several studies, including those by Bungartz *et al*. (1995) [4], Vieira *et al*. (2016) [24], Silva *et al*. (2017) [6], and Egashira *et al*. (2019) [9], which suggest the absence of an impact of ovarian super-stimulation on cumulus-oocyte complex (COC) recovery. This disparity may be attributed to various influencing factors, including vacuum pressure, needle diameter, operator proficiency, the donor animal's follicular population size, the donor animal's breed, pre-stimulation with hormones, the timing and frequency of Ovum Pick-Up (OPU) sessions, the synchronization of follicular waves, and individual variations among animals.

The developmental proficiency of oocytes, a pivotal determinant for *In-Vitro* Embryo Production (IVEP), is intricately associated with the quality of Cumulus-Oocyte Complexes (COCs) recovered. In this study, the average number of Grade 1 oocytes acquired per individual demonstrated an elevated value in the FSH-stimulated group (4.80 ± 0.64) in contrast to the non-stimulated group (1.30 ± 0.39). These outcomes align with the research conducted by Looney *et al*. (1994) [16], Perez *et al*. (2000) [19], Jeyakumar (2004) [14], Aller *et al*. (2012) [1], and Srimannarayana *et al*. (2019) [21], which reported an enhancement in the yield of high-quality oocytes following FSH stimulation prior to OPU.

This occurrence may be ascribed to the progressive enhancement in oocyte developmental competence with the augmentation of follicle size as it nears ovulation. Additionally, the administration of FSH could potentially

contribute to a more advantageous reorganization of oocyte cytoplasm, consequently leading to a heightened proportion of high-quality oocyte recovery. It is noteworthy that these present findings deviate from the results documented by Silva *et al*. (2017) [6], potentially influenced by individual donor peculiarities, breed divergences, lactation stage, physiological stressors, and the impact of environmental variables such as humidity, temperature, seasonality, and follicular population dynamics.

In this contemporary investigation, the proportions of cumulus cell expansion (93.32 ± 2.72 vs 91.01 ± 1.79) and first polar body extrusion (60.35 ± 2.05 vs 65.53 ± 1.86) demonstrated no statistically significant disparities ($p>0.05$) between the non-stimulated group and the FSH-stimulated group. The lack of significant differences in the extrusion of the first polar body, a key indicator of nuclear maturation in mammalian oocytes, may be attributed to potential oocyte degradation due to aging. It is noteworthy that in this study, oocytes were denuded post-*In Vitro* Fertilization (IVF), potentially contributing to a reduced visualization of polar body extrusion. These observations diverge from the outcomes elucidated by Donnay *et al*. (1997), and the discrepancies in *in vitro* maturation rates may be influenced by factors including animal genotype, follicle size and quality, potential oocyte damage during follicular aspiration, criteria employed for oocyte selection, and the composition of the *in vitro* maturation medium.

A pivotal factor contributing to the success of *in vitro* maturation lies in the formulation of the provided medium. The degree of cumulus cell expansion is influenced by the presence of gonadotropins and growth factors within the *in vitro* maturation media. Oocytes from the non-stimulated group may acquire the necessary elements for cumulus expansion and subsequent attainment of meiotic competency during *in vitro* maturation. While FSH stimulation preceding Ovum Pick-Up (OPU) significantly elevates the number of aspirated follicles and the oocyte recovery rate, it does not augment the capacity of oocytes to undergo cumulus expansion and first polar body extrusion.

Conclusıon

The outcomes of the present study suggest that the provision of follicle-stimulating hormone confers benefits in fostering the advancement of the aggregate follicular count and the presence of medium-sized antral follicles preceding ovum pick-up. Moreover, the supplementation of FSH elevates the quality of oocytes and enhances their proficiency for *in vitro* embryo production.

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