International Journal of Bio-resource and Stress Management https://ojs.pphouse.org/index.php/IJBSM

Crossref

IJBSM July 2024, 15(7): 01-07 **Article** AR5457

Research Article *Natural Resource Management*

DOI: HTTPS://DOI.ORG/10.23910/1.2024.5457

Revolutionizing Cattle Breeding: Studies on *In-vitro* Embryo Production in Sahiwal Cows with Sexed Semen

S. R. Farheen[1], G. Arunakumari[2], K. R. C. Reddy[3], K. C. S. Reddy[4], P. Nagaraj[5] and B. Priyanka[6]

[1]Dept. of Veterinary Gynaecology and Obstetrics, [3]Dept. of Veterinary Clinical Complex, [4]Dept. of Veterinary Gynaecology and Obstetrics, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana (500 030), India

[2]Dept. of Veterinary Gynaecology and Obstetrics, [5]Dept. of Veterinary Medicine, [6]Dept. of Veterinary Clinical Complex, College of Veterinary Science, Korutla, Jagitial, Telangana (505 326), India

Open Access

Corresponding shaikfarheen128@gmail.com

0009-0009-3927-2701

ABSTRACT

The experiment was conducted during 2023 (January–October), at Embryo transfer and *in-vitro* fertilization project, Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science, Korutla, Jagitial district, Telangana, *India*. The objective of the present study was to evaluate the effect of Follicle Stimulating Hormone stimulation on *In-Vitro* Fertilization rates with sexed semen *in-vitro* matured oocytes collected from Sahiwal cows via Ovum Pick-Up procedure. Irrespective of the estrus cycle, 20 non-lactating cows were divided into two equal groups. Animals in group 1 (non-stimulated, n=10) were subjected to ovum pick-up once at the random stage of the estrous cycle. Animals in group 2 (FSH Stimulated, n=10) were put under CIDR+FSH super stimulation protocol and subjected to OPU after 36 hours of coasting period. Viable oocytes were *in-vitro* fertilized using sexed-sorted semen after 24 hours of *in-vitro* maturation. Follicle stimulating hormone stimulation increased the secondary polar body extrusion rate (non-stimulated, 69.10±2.00% vs FSH stimulated, 79.84±1.55%), cleavage rate on day 1(non-stimulated, 60.69±3.04% vs FSH stimulated, 69.10±2.26%), cleavage rate on day 3 (non-stimulated, 35.62±3.32 vs FSH stimulated, 43.59±1.90), mean number of *in-vitro* produced blastocysts animal^{-1} (non-stimulated, 1.50±0.16 vs. FSH-treated, 4.10±0.23%) and blastocyst conversion rate (non-stimulated, 23.98±2.39% vs FSH-treated, 34.35±3.42%). We concluded that FSH stimulation was effective in improving *In-vitro* oocyte competence for embryo production in non-lactating Sahiwal cows with sexed semen.

KEYWORDS: Follicle stimulating hormone, sexed semen, blastocyst

***Citation* (VANCOUVER):** Farheen et al., Revolutionizing Cattle Breeding: Studies on In-vitro Embryo Production in Sahiwal Cows with Sexed Semen. *International Journal of Bio-resource and Stress Management*, 2024; 15(7), 01-07. HTTPS://DOI.ORG/10.23910/1.2024.5457.

Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

Conflict of interests: The authors have declared that no conflict of interest exists.

RECEIVED on 16th May 2024 RECEIVED in revised form on 28th June 2024 ACCEPTED in final form on 07th July 2024 PUBLISHED on 10th July 2024

1. INTRODUCTION

The Sahiwal cow is recognized as one of India's prominent milch breeds. This breed was traditionally bred in the regions of Ferozpur and Amritsar in Punjab, and the Sriganganagar district in Rajasthan. The Sahiwal breed is noted for its unique traits, including resistance to diseases and parasites, heat tolerance, and a high milk production range of 1600 to 2750 kg (Dahiya et al., 2024).

India is the world's leading milk producer, with 221.1 mmt of milk production, contributing 24% of the world's milk output (Anonymous, 2019). It is an agriculturally based country with a diverse animal population, with livestock as its most significant sideways link to farmers (Gulati and Juneja, 2022). However, when compared to nations of developed dairy industries like the United States (US) and New Zealand, the average milk production cow^{-1} is incredibly low in India. Major factors in Indian cattle's low milk output were (Dhara et al., 2006), lack of extensive genetic improvement programs, poor nutrition, poor farm management, inadequate veterinary y and extension services. Natural breeding could only achieve a small fraction of an elite individual's reproductive potential (Daly et al., 2020). The use of Assisted Reproductive Technology (ART) enables the exploitation of genetically superior germplasm (Mueller et al., 2022). The use of artificial insemination (AI) tool, one of ART, enables to exploit the vast numbers of sperm produced by genetically superior bulls by adopting frozen semen technique (Mohmad Shah et al., 2019), but the reproductive potential of the female has been found to be underexploited. Embryo transfer (ET) technology enables a genetically superior female to produce more offspring with superior genetic quality (Phillips and Jahnke, 2016). In cattle, embryos are produced via multiple-ovulation embryo transfer (MOET) or *in-vitro* embryo production (IVEP). A greater number of transferrable embryos $donor^{-1}$ can be produced by IVEP than through MOET, reduced number of sperm is required for embryo production than in MOET and there is an increase in chance of obtaining the desired sex of offspring through IVEP (Baruselli et al., 2021). Furthermore, IVEP enables the production of embryos from a variety of sources, including open cyclic heifers, cows, animals that are unresponsive to super-ovulatory treatments, those with reproductive tract abnormalities, and older animals. Similarly, it can be applied to pregnant animals in their first trimester, postpartum cows, and prepubertal calves (Ferre et al., 2020). IVEP involves collecting immature oocytes from living cattle, followed by *in-vitro* maturation, fertilization, and culture, and then transferring the embryos to recipient females (Bo and Mapletoft, 2018).

Livestock owners in general desire to have female calves. Considering the need for replacement heifers in herds, increase in monetary value for heifer claves over bull calves (Nor et al., 2015), it is no wonder that dairy producers are interested in options that allow them to predetermine the sex of their calves. Combining IVF with sexed-sorted sperm is the advisable biotechnological strategy for obtaining offspring of predetermined sex to improve the productivity in farms (Xu et al., 2009). In IVEP, IVF with sexed semen lowers the cost of progeny testing programs.

Sexed semen was recently introduced in India, and its use in OPU-IVEP programs requires further standardization. Fine-tuning the technique, including FSH super stimulation before OPU and using sex-sorted semen, is needed for large-scale embryo production, so, this research was carried out with the objective, to study the effect of FSH stimulation prior to OPU on *in-vitro* fertilization rates of Sahiwal cow oocytes with sex-sorted semen.

2. MATERIALS AND METHODS

Present study was conducted at Embryo transfer and *in vitro* Fertilization (ET and IVF) project, Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science, Korutla, Jagitial district, Telangana, India (latitude: 18° 49′36.71" N; longitude: 78° 42′ 50.39" E; altitude: 295.99 m above mean sea level) during the period between January to October, 2023. Twenty Sahiwal cows aged 3–6 years and weighing between 250 to 450 kg body weight were selected as oocyte donors. Irrespective of the estrus cycle, cows were divided into two equal groups. Animals in group 1 (non-stimulated, n=10) were subjected to OPU once at the random stage of the estrous cycle. Whereas cows in group 2 (FSH Stimulated, n=10) were subjected to super stimulation with FSH and OPU was carried out 36hrs after the last FSH injection (coasting period) (Farheen et al., 2024). The recovered cumulus-oocyte complexes were evaluated and kept for *in-vitro* Maturation for 24 hours. After 24 hours the *in-vitro* matured oocytes were subjected to *In-vitro* fertilization with sexed semen.

Before conducting *In-vitro* Fertilization (IVF), to IVF medium specific aliquots of components (H and PHE) were added. This mixture was used to prepare IVF dishes, where each well was filled with 500 µl of IVF media topped with mineral oil. The dish was then placed in a benchtop incubator (humidified atmosphere of 6% CO_2, 5% O_2, and 89% N_2) overnight for equilibration. On the day of IVF, matured oocytes were washed 2–3 times with prepared IVF media and transferred into the pre-prepared IVF media in the dish. The dish containing the oocytes was kept in the incubator until the semen preparation was completed.

In this study, sex-sorted frozen sperm was utilized at a concentration of 2×10^6 sperm 0.25 ml^{-1} straw. Thawing

was conducted in a machine at 37°C for 30 seconds, and the resulting post-thaw progressive motility was 40%. The thawed semen was then subjected to a centrifugation (2400 G for 6 min) process using a pre-warmed gradient for selection of highly motile spermatozoa. Immediately after centrifugation, the supernatant formed was removed and to this prepared IVF media was added and kept for second centrifugation at 600 G for 3 min. After centrifugation the supernatant formed was removed leaving semen with motile spermatozoa. Semen of approx. 10–20 µl was inseminated into IVF media containing matured oocytes. The IVF dish was placed in a benchtop incubator for approximately 16–18 hours. Following this incubation period, the fertilization status of the oocytes was assessed based on various criteria such as 2nd polar body extrusion, changes in oocyte shape, and cleavage on day 1 (Figure 1).

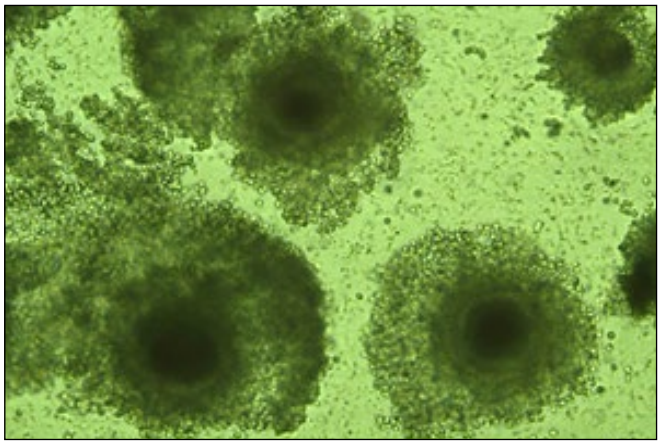

Figure 1: Co-incubation of cumulus Oocyte complexes and sex sorted spermatozoa

2.1. *In-vitro culture (IVC) of presumptive zygotes*

Following a 16–18 hours co-incubation of gametes, presumptive zygotes were transferred to freshly prepared drops of preheated washing media. Mechanical denudation of cumulus cells from putative zygotes was achieved using a denudation pipette in the washing media. The denuded zygotes underwent multiple washes with both the washing media and equilibrated IVC media before being transferred to IVC dishes. These dishes were placed in a benchtop incubator (5% CO_2, 5% O_2, 90% N_2, 38.5°C temperature and more than 90% RH) for six days. Cleavage was assessed on days 3 and 7, and the rates were recorded. Additionally, the number of blastocysts formed and the blastocyst conversion rate were recorded on day 7.

2.2. *Statistical analysis*

The data collected was analyzed statistically using descriptive statistics and the means were tested for significance by students T-test using SPSS (2009) version 16.

3. RESULTS AND DISCUSSION

In the present experiment, the *in-vitro* fertilization rate is estimated by 2nd polar body extrusion into peri vitelline space, cleavage rates on day 1 of *in-vitro* culture (IVC), cleavage rates on day 3 of IVC, number of blastocysts produced on day 7 of IVC and blastocyst conversion rate on day 7 of IVC (Table 1 and Figure 2).

Table 1: Effect of FSH stimulation on *in-vitro* fertilization rates of oocytes collected from non-stimulated and FSH stimulated Sahiwal cows with sexed semen

	Non-stimulated group	FSH stimulated group
No. of oocytes kept for insemination	58	129
No. of oocytes showing second polar body extrusion	40	97
The mean number of oocytes showing 2nd polar body extrusion cow^{-1}	4.00±0.14	9.70±0.90*
Second polar body extrusion rate (%)	69.10±2.00	79.84±1.55*
Number of oocytes showing cleavage on day 1	35	90
The mean number of oocytes showing cleavage on day 1 cow^{-1}	3.5±0.16	9.00±1.00*
Cleavage rate on day 1 of IVC (%)	60.69±3.04	69.10±2.26*
Number of oocytes showing cleavage rate on day 3	21	55
The mean number of oocytes showing cleavage on day 3 cow^{-1}	2.10±0.23	5.50±0.45*
Cleavage rate on day 3 of IVC (%)	35.62±3.32	43.59±1.90*
Number of blastocysts formed on day 7	15	47
The mean number of blastocysts produced animal^{-1} on day 7 of IVC	1.50±0.16	4.70±0.33*
Blastocyst conversion rate (%) on day 7 of IVC	23.98±2.39	38.10±2.69*

Mean number of oocytes showing 2nd polar body extrusion, cleavage on day 1 of IVC, cleavage on day 3 of IVC, 2nd polar body extrusion rate, cleavage rate on day 1 of IVC, cleavage rate on day 3 of IVC, blastocyst conversion rate within row differs significantly (p<0.05)

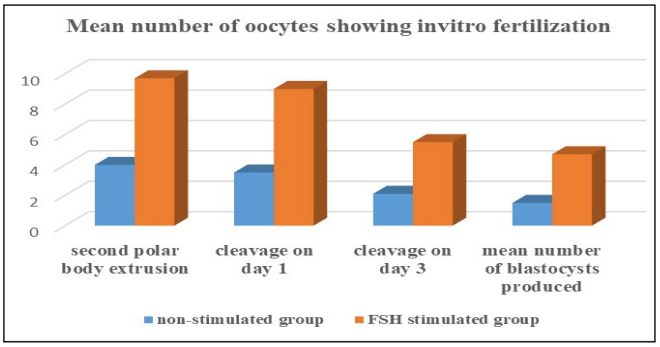

Figure 2: Mean number of oocytes per cow showing second polar body extrusion into perivitelline space, cleavage on day 1, cleavage on day 3 and mean number of blastocysts producer

The second polar body extrusion rate (non-stimulated-69.10±2.00, FSH stimulated 79.84±1.55), cleavage rate on day 1 (non-stimulated 60.69±3.04, FSH stimulated 69.10±2.26), cleavage rate on day 3 (non-stimulated 35.62±3.32, FSH stimulated 43.59±1.90) were significantly higher (p<0.05) in the FSH stimulated group than in the non-stimulated group, this may be because FSH stimulation before OPU causes *in-vivo* maturation of oocytes that have superior developmental competence compared to completely *in-vitro* matured oocytes (Hendriksen et al., 2000 and Humblot et al., 2005). The cleavage rate of *in-vitro*-produced presumptive zygotes from the non-stimulated group utilizing sex-sorted semen is relatively lower in Sahiwal cows in the present study (60.69±3.04) than those presented by Pontes et al. (2011) 78% in Holstein cows, 68% in Gir cows, Matoba et al. (2014) 75.4±5.2% in Holstein cows, Nogueira et al. (2021) observed an average cleavage rate of 81.6%, this variation in results may be due to the difference in breed selected, frequency of OPU session performed per donor, age of donor and seasonality effect (Figure 3 and 4).

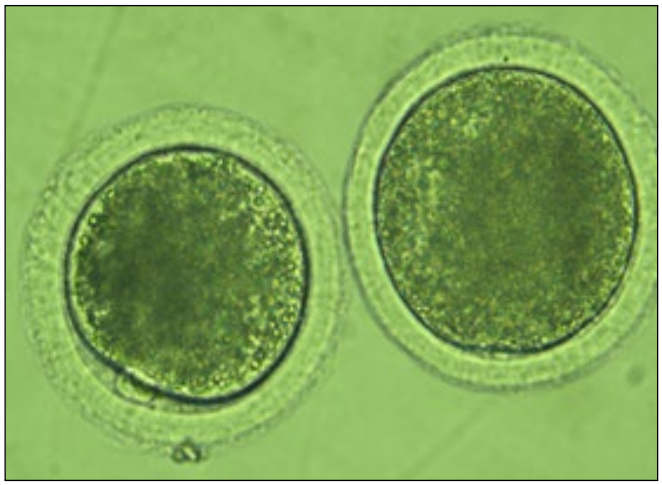

Figure 3: Extrusion of second polar body in to perivitelline space after IVF (under 20×of phase contrast microscope)

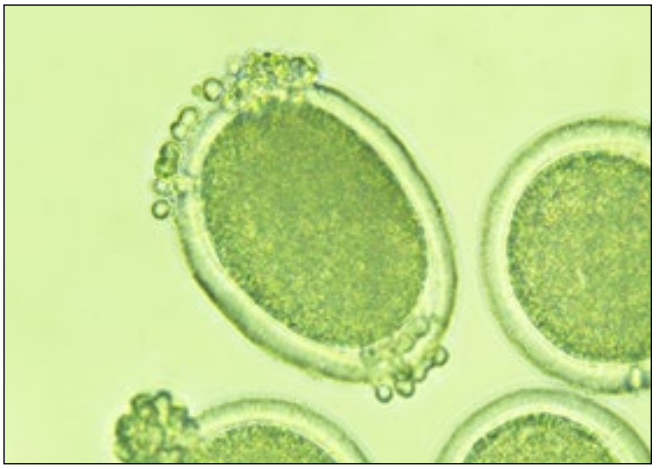

Figure 4: Change in shape of oocyte after IVF (under 20×of phase contrast microscope)

The *in-vitro* produced blastocyst rate in the present study is significantly higher in FSH stimulated group (38.10±2.69%) than non-the stimulated group (23.98±2.39%). These results are in accordance with Giorgio et al. (2010) reported higher rate of blastocyst development in stimulated when compared to non-stimulated animals by using sexed semen (25.2% versus 12.8%, p=0.001). The higher blastocyst rate in FSH stimulated group in the present study may be due to the availability of good-quality oocytes for culture. The rate of normal cortical granule (CG) distribution in the cytoplasm are higher in good quality oocytes which are higher in the super stimulated group than in the non-stimulated group (Egashira et al., 2019). The CGs are distributed near the plasma membrane of the oocytes during final maturation. Only CGs that exist very close to the plasma membrane can fuse and release their contents into the perivitelline space. Exocytosis of CGs is very important in the prevention of polyspermy and thereby increase the number of oocytes fertilized, cleavage rate and blastocyst rate (Figure 5 to 15).

The findings of *in-vitro* produced blastocyst rates with sex sorted semen in non-stimulated group are reported higher by Pontes et al. (2011) 46%, Matoba et al. (2014) 31.8±8.2% in Holstein cows, Demetrio et al. (2020) 27% and Nogueira et al. (2021) 30.08% in cows than blastocyst rate (23.98±2.39%) by using sex sorted semen) recorded

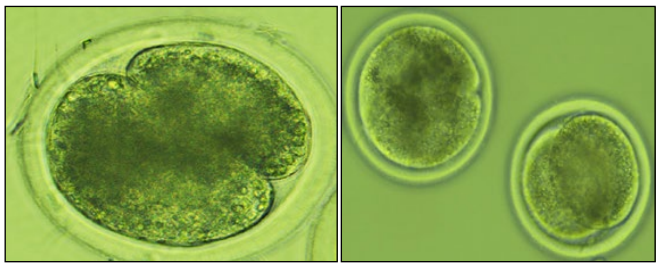

Figure 5: Two celled embryos (under 20×of phase contrast microscope)

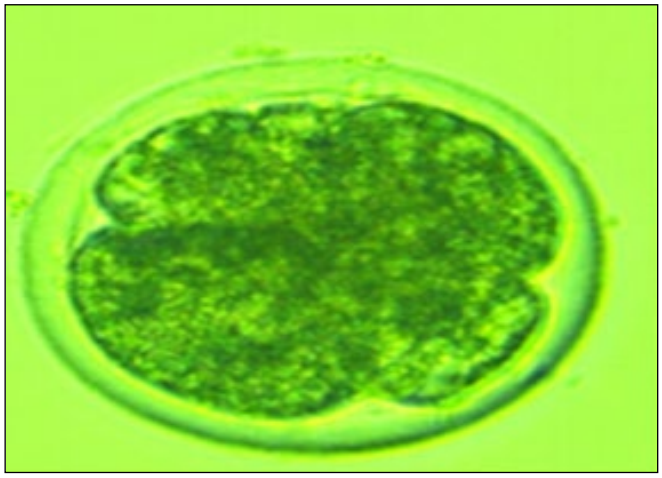

Figure 6: Four celled embryos

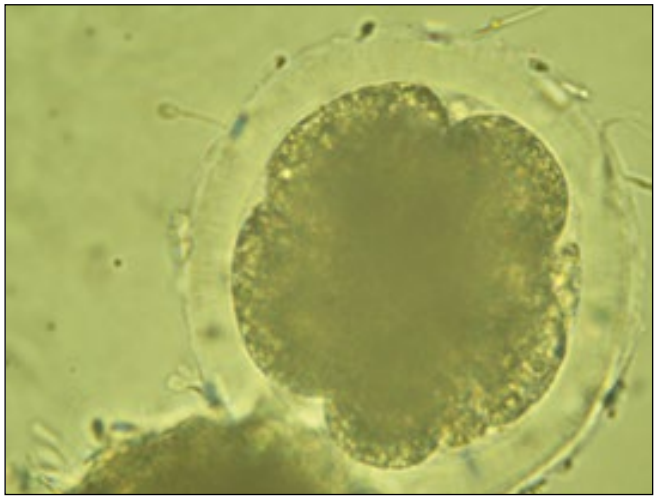

Figure 7: Eight celled embryos

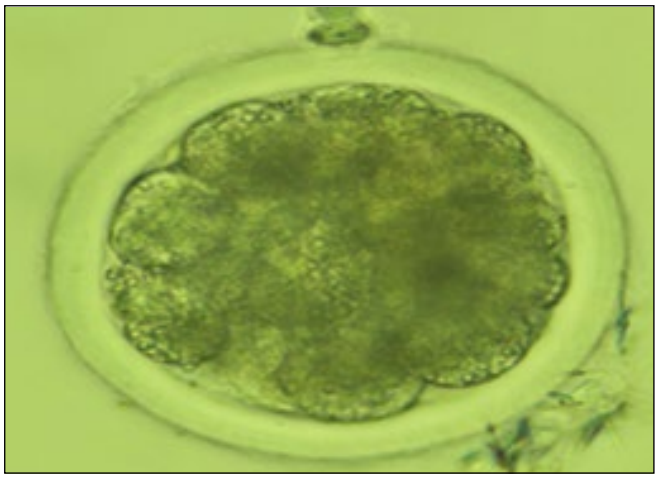

Figure 8: Sixteen celled embryos (under 20×of phase contrast microscope)

in the present study. This variation may be due to the difference in breed selected, frequency of OPU session performed, age of donor.

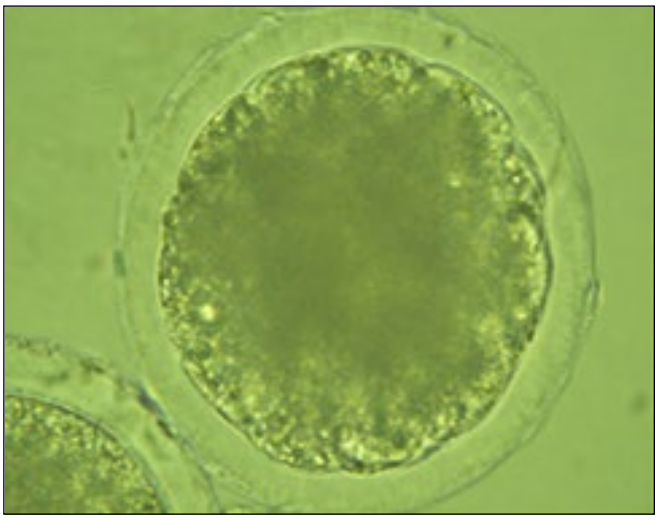

Figure 9: Stage 4 or Morula stage of embryo (under 20×of phase contrast microscope)

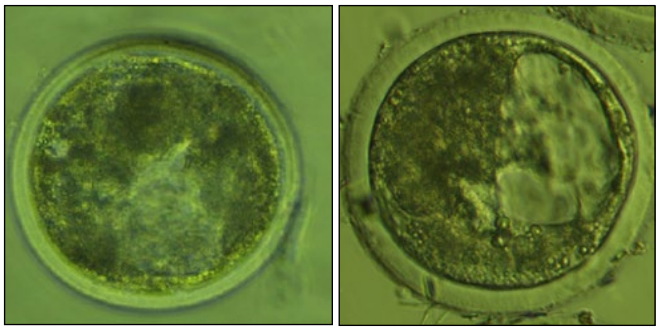

Figure 10: Early blastocyst or stage grade 5 of embryo (under 20×of phase contrast microscope)

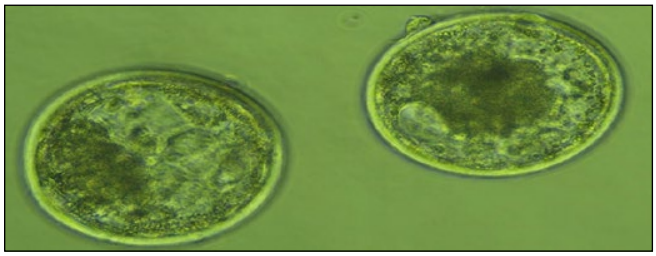

Figure 11: Blastocyst or Stage 6 grade of embryo (under 20×of phase contrast microscope)

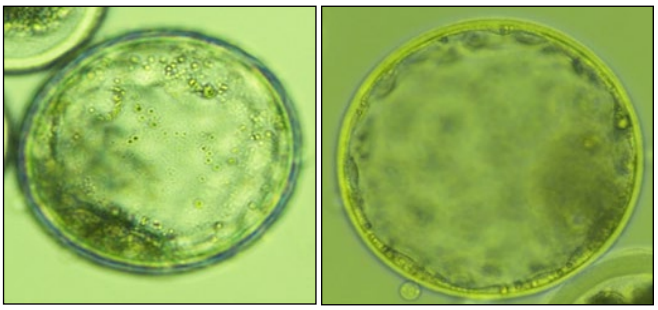

Figure 12: Expanded blastocyst or stage 7 grade (under 20×of phase contrast microscope)

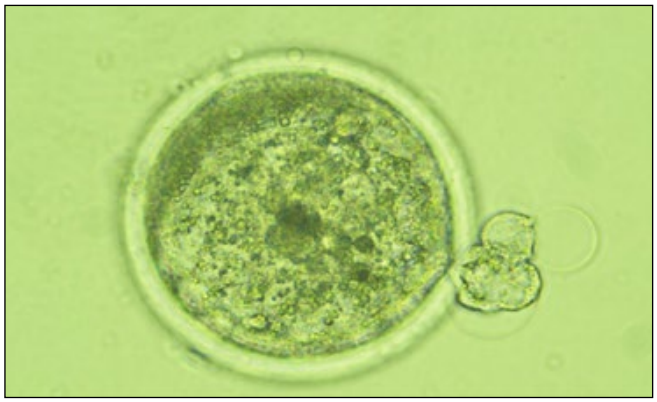

Figure 13: Hatching blastocyst or stage 8 grade of embryo (under 20×of phase contrast microscope)

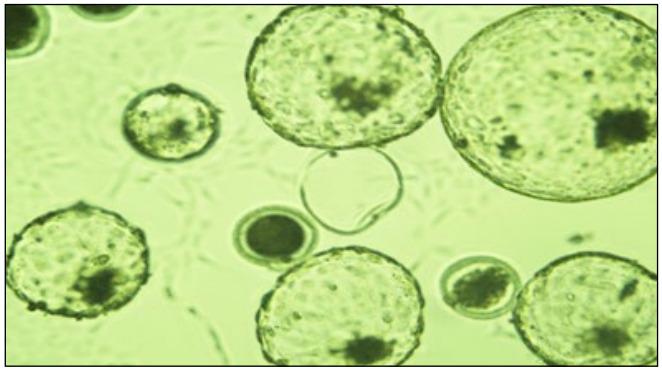

Figure 14: Hatched blastocyst or stage 9 grade embryo (under 20×of phase contrast microscope)

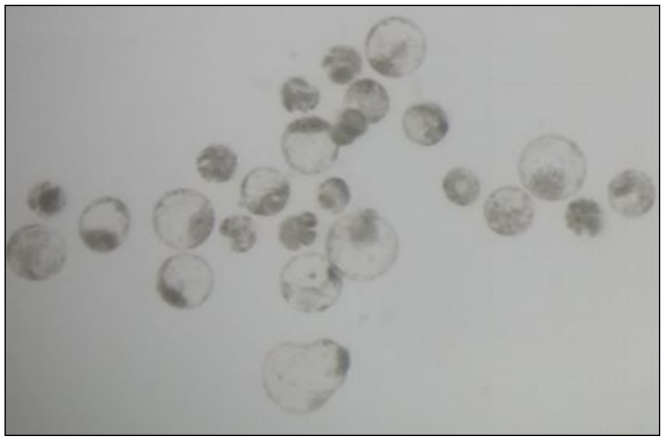

Figure 15: Different stages of embryos in one experiment on day 7 of IVC (under 10×of stereo zoom microscope)

The findings of *in-vitro* produced blastocyst rates on day 7 as a result of sex sorted semen in FSH stimulated group are reported higher by Matoba et al. (2014) 45.7±9.3% in Holstein cows, this may be due to breed difference, different super ovulatory protocol used (CIDR+Dominant follicle ablation (DFA)+injection FSH) and he concluded that DFA causes increase in number of *in vivo* matured oocytes in OPU, thereby increases the number of good quality blastocyst produced by IVF using sex sorted semen.

4. CONCLUSION

FSH stimulation was effective in improving *In-vitro* oocyte competence for embryo production in non-lactating Sahiwal cows with sexed semen. *In-vitro* embryo production utilizing sex-sorted sperm is a feasible means to produce calves of pre-selected sex.

5. ACKNOWLEDGEMENT

I would like to express my deepest gratitude to my advisor mam, Dr G Aruna Kumari, for their invaluable guidance and support throughout my research process.

6. REFERENCES

Anonymous, 2019. Crop Statistics 2019. FAO of UN, International Fertilizer Industry Association. Available from http://www.fao.org/faostat/en/#data/QC. Accessed on 20 December, 2020.

Baruselli, P.S., Rodrigues, C.A., Ferreira, R.M., Sales, J.N.S., Elliff, F.M., Silva, L.G., Viziack, M.P., Factor, L., Michael, J.D., 2021. Impact of oocyte donor age and breed on in vitro embryo production in cattle, and relationship of dairy and beef embryo recipients on pregnancy and the subsequent performance. Reproduction Fertility and Development 34(2), 36–51.

Bhandari, G., Chandel, B.S., Bhakat, M., 2020. Cost of conservation of indigenous dairy cattle breed-a case of Sahiwal in India. Indian Journal of Animal Sciences 90(10), 1392–1397.

Bo, G.A., Mapletoft, R.J., 2018. Embryo transfer technology in cattle. Animal Biotechnology 1: Reproductive Biotechnologies, 107–133.

Dahiya, N.K., Bajaj, S.B., Ruhil, A., Jaglan, V., 2024. Udderly accurate: A deep learning-based modelling for determination of dairyness of Sahiwal cow using computer vision. The Indian Journal of Animal Sciences 94(1), 83–87.

Daly, J., Smith, H., McGrice, H.A., Kind, K.L., William, H.E.J., van Wettere, 2020. Towards improving the outcomes of assisted reproductive technologies of cattle and sheep, with particular focus on recipient management. Animals 10(2), 293.

Demetrio, D.G.B., Benedetti, E., Demetrio, C.G.B., Fonseca, J., Oliveira, M., Magalhaes, A., Santos, R.M., 2020. How can we improve embryo production and pregnancy outcomes of Holstein embryos produced *in vitro*? Animal Reproduction 17, e20200053.

Egashira, J., Ihara, H., Khatun, Y., Wada, T., Konno, Y., Tatemoto, H., Yamanaka, K.I., 2019. Efficient *in vitro* embryo production using *in vivo*-matured oocytes

from superstimulated Japanese Black cows. Journal of Reproduction and Development 65, 183–190.

Farheen, S.R., Kumari, G.A., Reddy, K.R., Reddy, K.C., Nagaraj, P., Priyanka, B., 2024. Effect of follicle stimulating hormone stimulation on *In-vitro* oocyte maturation in Sahiwal cattle. International Journal of Advanced Biochemistry Research 8(3), 111–117.

Ferre, L.B., Kjelland, M.E., Strøbech, L.B., Hyttel, P., Mermillod, P., Ross, P.J., 2020. Recent advances in bovine in vitro embryo production: reproductive biotechnology history and methods. Animal 14(5), 991–1004.

Giorgio, F., Enright, B., Rizos, D., Boland, M., Lonergan, P., 2010. Optimization of *in vitro* bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. Theriogenology 57, 2105–2117.

Gulati, A., Juneja, R., 2022. Transforming Indian agriculture. Indian Agriculture Towards 2030, 9–37.

Hendriksen, P.J.M., Vos, P.L.A.M., Steenweg, W.N.M., Bevers, M.M., Dieleman, S.J., 2000. Bovine follicular development and its effect on the *in vitro* competence of oocytes. Theriogenology 53, 11–20.

Humblot, P., Holm, P., Lonergan, P., Wrenzycki, C., Lequarre, A.S., Joly, C.G., Herrmann, D., Lopes, A., Rizos, D., Niemann, H., Callesen, H., 2005. Effect of stage of follicular growth during superovulation on developmental competence of bovine oocytes. Theriogenology 63, 1149–1166.

Matoba, S., Yoshioka, H., Matsuda, H., Sugimura, S., Aikawa, Y., Ohtake, M., Imai, K., 2014. Optimizing production of *in vivo*-matured oocytes from super stimulated holstein cows for *in vitro* production of embryos using X-Sorted sperm. Journal of Dairy Science 97(2), 743–753.

Mohmad, S.S., Manmohan, C., 2019. Modern biotechnological tools for enhancing reproductive efficacy in livestock. Indian Journal of Genetics and Plant Breeding 79(1), 241–249.

Mueller, M.L., Van, E.A.L., 2022. Synergistic power of genomic selection, assisted reproductive technologies, and gene editing to drive genetic improvement of cattle. CABI Agriculture and Bioscience 3(1), 13.

Nogueira, B.G.R., de Souza, L.F.A., Puelker, R.Z., Giometti, I.C., Firetti, S.M., Dias, T.S.D.S.B., Castilho, C., 2021. Factors affecting the *in vitro* production of bovine embryos in a commercial program. Research, Society and Development 10(2), e16110212264–e16110212264.

Nor, N.M., Steeneveld, W., Mourits, M.C.M., Hogeveen, H., 2015. The optimal number of heifer calves to be reared as dairy replacements. Journal of Dairy Science 98(2), 861–871.

Phillips, P.E., Jahnke, M.M., 2016. Embryo transfer (techniques, donors, and recipients). Veterinary Clinics: Food Animal Practice 32(2), 365–385.

Pontes, H.F., Sterza, F.M., Basso, A.C., Ferreira, C.R., Sanches, B.V., Rubin, K.C.P., Seneda, M.M., 2011. Ovum pick up, *in vitro* embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (*Bos indicus*) donors. Theriogenology 75(9), 1640–1646.

Xu, J., Chaubal, S., Du, F., 2009. Optimizing IVF with sexed sperm in cattle. Theriogenology 71(1), 39–47.