Role of Cryoprotectants in Preventing Quality Loss of Goat Oocytes after Warming by Examining Insulin-Like Growth Factor-1 and Bone Morphogenetic Protein-15 Expression

Zahra Shabira[1], Widjiati[2]*, Rimayanti[2], Tita Damayanti Lestari[2], Erma Safitri[2], Maslichah Mafruchati[2], Viski Fitri Hendrawan[3]
[1]Postgraduate Student of Reproductive Biology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia, [2]Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia, [3]Faculty of Veterinary Medicine, Universitas Brawijaya, Malang, East Java, Indonesia.
*Corresponding author: widjiati@fkh.unair.ac.id

Abstract

This research aimed to assess the efficacy of ethylene glycol-sucrose as an alternative cryoprotectant compared to commercial cryoprotectants by focusing on the expression of insulin-like growth factor-1 (IGF-1) and bone morphogenetic protein-15 (BMP-15) in goat oocyte cumulus complexes after warming with identification using the immunocytochemical analysis. Maturated oocytes were divided into 3 groups, i.e., (C) the control group followed by an immunocytochemical examination, and two treatment groups, i.e., (P1) was exposed to a commercial cryoprotectant for 15 minutes, then placed in a hemistraw and dipped in liquid nitrogen, and (P2) was exposed to 30% ethylene glycol and 1 M sucrose for 15 minutes, then placed in a hemistraw and dipped in liquid nitrogen. The results revealed divergent responses in IGF-1 (CG = 9.00 ± 3.00; P1 = 9.50 ± 3.20; P2 = 4.67 ± 0.94) and BMP-15 (CG = 10.50 ± 3.35; P1 = 9.50 ± 2.69; P2 = 5.50 ± 3.64) expression patterns, possibly influenced by the two cryoprotectant abilities and oocytes performing their permeabilities to cryoprotectant solution. We concluded that each cryoprotectant is necessary to mitigate the adverse impact on gene expression in oocytes so that we can use it for reproductive technologies.

Keywords: BMP-15, food availability, goat oocyte, IGF-1, post-warming

Received: June 21, 2024 **Revised:** November 4, 2024 **Accepted:** January 26, 2025

INTRODUCTION

Biotechnology aims to improve animal reproduction efficiency, particularly by using oocytes for the generation of embryos while accounting for the high production of superior genetics and food economic requirements (Nemer *et al*., 2018). The process of egg maturation in vitro requires nourishment and the presence of steroidogenesis activity for a successful in vitro fertilization (IVF) operation. Steroidogenesis activity is regulated by proteins like bone morphogenetic protein-15 (BMP-15) and insulin-like growth factor-1 (IGF-1) (Dai *et al*., 2022; Bahari *et al*., 2023).

The choice of the right cryoprotectant that won't harm the goat oocytes is important to preserving their quality during the vitrification and warming phases (Chang *et al*., 2022). The maturation gene factors, BMP-15 and IGF-1, were found through an immunocytochemistry stain procedure. Multiple previous studies have shown that ethylene glycol (EG) and sucrose can maintain cow and pig oocytes and vitrify goat oocytes, subsequently offering valuable insights for future reproductive biotechnology techniques. When combined with sucrose, which functions as an extracellular cryoprotectant and binds to the lipid layer of cell membranes to protect membrane proteins from shock, EG can rapidly permeabilize, protecting oocytes and embryos before vitrification (Campbell and Brockbank, 2022) and also supplies energy for the metabolism of thawed sperm and warming oocytes (Herdis *et al*., 2019). Enhancement of ovarian tissue quality and increased vitrification output of sheep, buffalo, and cow oocytes has been demonstrated by intriguing research employing EG and sucrose (Borges *et al*., 2018; Prastiya *et al*., 2019).

Even with advancements, selecting the right cryoprotectant is crucial to preserving oocyte viability and reducing toxicity during

vitrification, particularly since no appropriate cryoprotectant has been discovered for goat oocytes (Best, 2015; Juliawan *et al*., 2022). Thus, the purpose of this study is to assess EG-sucrose's effectiveness as a substitute for commercial cryoprotectants. We anticipate that when compared to commercial cryoprotectants, EG, and sucrose will be more cost-effective due to their greater availability, ease of production, and infinite volume use of alternatives, which will support the availability of food, particularly animal-derived food.

MATERIALS AND METHODS

Ethical Approval

We carried out this research under the ethical clearance designation from the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, with registration No.1.KEH.051.03.2023.

Study Period and Location

This study employed a completely randomized design approach utilizing goat oocytes cross-out from the ovaries of local goats procured from a slaughterhouse. The process of vitrification was carried out, and data analysis took place in vitro at the Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia from January to July 2023.

Cryopreservation Media Formulation

The cryopreservation media was formulated for oocyte collection, oocyte maturation, in vitro fertilization, and in vitro culture. Subsequently, the cryopreservation media was distributed in the form of droplets and placed in a 5% CO_2 incubator at a temperature of 38.5°C one day before oocyte collection (Krause *et al*., 2022).

Oocyte Collection

Goat ovaries were procured from a slaughterhouse and then transported to the laboratory inside a flask filled with 0.95% NaCl at 37°C. At the laboratory, the ovaries were taken off the hanging device, meticulously cleaned, and rinsed with PBS 5% and 100 µL of gentamycin until they were entirely clear. Oocyte retrieval was carried out through aspiration using a syringe equipped with an 18G needle containing 1 mL of Universal IVF Medium (Ref. 10300060) media. Following this, the gathered oocytes were placed in a sterile Petri dish and examined under a microscope. Only oocytes surrounded by cumulus complexes with more than 3 layers are chosen for the in vitro maturation process (Hardy *et al*., 2000).

In Vitro Maturation

Oocytes were collected from goat ovaries, which were surrounded with 2 or 3 layers of cumulus cells flushing about 3 times using Syn Vitro Flush with heparin (10 IU/mL) (Ref. 15750125A) from ONGIO. After that, oocytes were harvested in Universal IVF Medium (Ref. 10300060) from ONGIO and incubated for 22 hours at 38.5°C with 5% CO_2. Matured oocytes were selected under the microscope and the best-quality oocyte cumulus complex was for the vitrification procedure (Widjiati *et al*., 2011).

Vitrification Treatment

We used a vitrification procedure that has been adapted and established from RI Patent No. IDP000086462. Maturated oocytes were divided into 3 groups, i.e., (C) the control group followed by an immunocytochemical examination, (P1) was exposed to commercial cryoprotectant for 15 minutes and then placed in a hemistraw and dipped in liquid nitrogen, (P2) was exposed to 30% EG and 1 M sucrose for 15 minutes then placed in a hemistraw and dipped in liquid nitrogen. Vitrification of the two groups of oocytes was allowed for 7 days (Ahuja and Macklon, 2020).

Warming Treatment of Vitrified Oocytes

Two types of warming cryoprotectants were utilized a commercial warming kit and a modified warming kit containing sucrose at concentrations of 0.25 M, 0.5 M, and 1 M. Then, we retrieved two hemistraws containing each oocyte treatment group from liquid nitrogen after days, and each hemistraw was exposed to the commercial warming solution and the modified warming

solution for about 10 minutes. Following the warming process, the oocytes were transferred again into fresh Universal IVF Medium (Ref. 10300060) (Doyle *et al*., 2016).

Immunocytochemistry

Immunocytochemicals initiated by moving the grouped oocytes to a sterile glass object, covering it with a sterile cover glass, placing it in a closed container, and soaking it in a solution of methanol and acetic acid (ratio 1:3) for a whole day. Subsequently, the glass object sample was extracted and cleaned three times with 10% PBS. Next, 3% H2O2 was added and allowed to sit for ten minutes. Finally, BMP-15 (Polyclonal Antibody Bioss) and IGF-1 (Antibody H-9, Ref. SC-518040) from Santa Cruz Biotechnology were included. Sterile glass objects were subjected to an hour-long magnetic immunostaining incubation, followed by a 5-minute tris buffer rinse, a 10-minute addition of 3,3-diaminobenzidine (DAB), a 5-minute sterile water rinse, and a 2-minute soaking in methylene green (Seyour *et al*., 2019; Novus Biologicals, 2021).

Data Analysis

The collected data from each group after the Remeley index scale evaluation were analyzed using the Kruskal-Wallis test, followed by the Mann-Whitney test using SPSS update version 22.

RESULTS AND DISCUSSION

The results of the immunocytochemical analysis revealed the expression of IGF-1 (Figure 1) and BMP-15 (Figure 2) in post-warming goat oocytes. Fresh oocytes and 2 vitrified oocytes showed colorization from immunocytochemistry using antibodies of IGF-1 and BMP-15, which the immunoreaction indicated with a brownish color visualized from respective figures.

The results indicate that there was no significant difference in IGF-1 expression between the C group (9.00 ± 3.00) and P1 group (9.50 ± 3.20) treated with commercial cryoprotectant, but there were significant differences observed between the P2 group (4.67 ± 0.94) and both of these groups. Furthermore, the subsequent results of BMP-15 expression show that there was no significant difference between the C group (10.50 ± 3.35) consisting of mature oocytes not subjected to vitrification, the P1 group (9.50 ± 2.69) mature oocytes vitrified with commercial cryoprotectant, and the P2 group (5.50 ± 3.64) comprising mature oocytes vitrified with 30% EG + 1 M sucrose (Table 1).

We suggest that the potential fluctuation in gene expression depends on the protocol used or the varied responses among genes affected by different cryoprotectants, resulting in individual oocyte variability (Andreis *et al*., 2021; Campbell and Brockbank, 2022).

The composition of commercial cryoprotectants typically consists of a mixture of dimethyl sulfoxide (DMSO) with EG, supplemented with trehalose and HEPES-buffered medium (Awan *et al*., 2020). The combination of DMSO and EG is commonly used due to its rapid permeability into the cumulus-oocyte complex (COCs) membrane, strong bonding with hydrogen and water, and non-potent nature (Sanaei *et al*., 2018; Safitri *et al*., 2022). The DMSO with trehalose also results in higher expression of genes involved in protein refolding, indicating that it enables certain recovery processes to counter freezing-warming damage in the cumulus-oocyte complex (Whaley *et al*., 2021). The advantage of using trehalose is its rapid binding to the α-1-1-glycoside group at extreme temperatures, reducing acid hydrolysis at low pH, and preserving gene factors in the cumulus-oocyte complex after warming (Zhang *et al*., 2019). Previous research testing the modification of 30% EG and 1 M sucrose found that this cryoprotectant can maintain the viability of bovine cumulus-oocyte complexes. This is because EG can penetrate more easily into the COCs, and sucrose can keep the COCs lipid membrane stable (Herdis *et al*., 2019; Juliawan *et al*., 2022). It has been well-demonstrated that EG-sucrose has successful survival rates for vitrification protocols (Azari *et al*., 2017; Hanifah *et al*., 2020).

Table 1. Remeley index scale to oocyte viability after seven days of vitrification

Groups	N	IGF-1 expression	BMP-15 expression
(C) Control groups	6	9.00 ± 3.00[a]	10.50 ± 3.35[a]
(P1) Commercial cryoprotectant	6	9.50 ± 3.20[a]	9.50 ± 2.69[a]
(P2) 30% EG + 1 M sucrose	6	4.67 ± 0.94[a]	5.50 ± 3.64[b]

Different superscripts indicate a significant difference ($p < 0.05$).

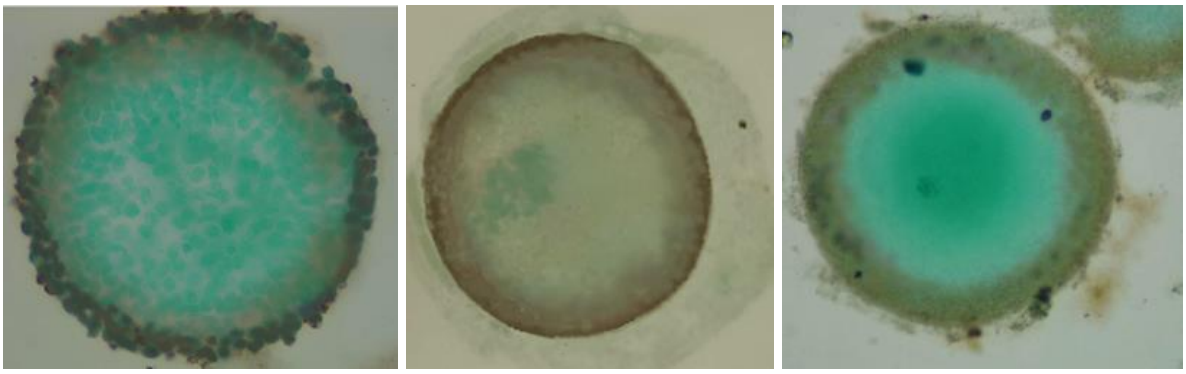

Figure 1. The expression of IGF-1 following oocyte warming for each treatment. (Immunocytochemistry, 400×).

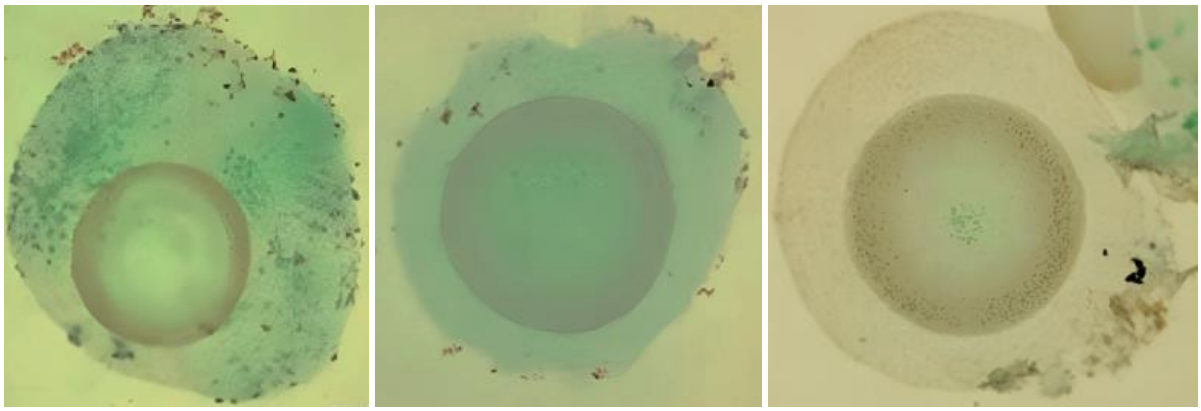

Figure 2. The expression of BMP-15 following oocyte warming for each treatment. (Immunocytochemistry, 400×).

The relationship between cryoprotectants and the cumulus-oocyte complex involves the permeability system, which includes the sensitivity of the cumulus-oocyte complex and cryoprotectant diffusion. In the cell permeability system, the mechanism of water-other fluid and the cell membrane is referred to as Lp (water permeability coefficient) and Ea (Arrhenius activation energy). This explains that if the Lp of the intracellular fluid is smaller than the high incoming Ea energy, then the external fluid can enter through simple diffusion across the cell membrane. Conversely, if the Lp of the intracellular fluid is higher than the low incoming Ea energy, then the external fluid must enter not only through simple diffusion but also through the aquaporin 3 channel. This applies to the mechanism of EG and DMSO fluids entering oocytes and cumulus cells. The differences between EG and DMSO can become toxic to the cumulus-oocyte complex for several reasons, as follows: (1) EG has a smaller molecular weight than DMSO at 6.78 g/mol, but if the concentration is not controlled and the equilibration time exceeds a certain period, the cumulus-oocyte complex can corrode; (2) DMSO is similar to EG, but it is known that there are specific conditions that cause DMSO to open channels other than aquaporin (Awan *et al*., 2020); and (3) if EG is mixed with DMSO, the permeability will not be

severe because EG breaks down DMSO into polar groups, which then bind with hydrogen and water molecules (Edashige, 2016; Borges *et al*., 2018).

Our further discussion is that cumulus-oocyte complex communication is a vital process for the development of competent oocytes, resulting from all gene factors secreted by the oocyte having a direct effect on cumulus cells. We obtained several references related to this research where maturation factor biomarkers were examined in COCs of various animal species after exposure to cryoprotectants and vitrification (Llonch *et al*., 2021). GDF-9 and BMP-15 were known to be high in bovine COCs exposed to 15% EG + 15% DMSO + 0.5 M sucrose and 40% EG + 1 M sucrose compared to fresh COCs (Azari *et al*., 2017). GDF-9 and BMP-15 were low in sheep COCs vitrified using 15% EG + 15% DMSO compared to fresh sheep COCs (Mishra *et al*., 2016). GDF-9 and BMP-15 had equivalent levels in bovine COCs vitrified using 20% EG + 20% DMSO and then stored in open-pulled straws (Chen *et al*., 2014). GDF-9 and BMP-15 were high in goat COCs exposed to 20% EG + 20% DMSO and then stored in conventional straws. The effect of EG and DMSO when combined with sucrose showed good integrity for the cumulus-oocyte complex after warming. It can also be known that the pattern of differences in gene expression is likely due to the reflection of the type of vitrification protocol or the sensitivity of COCs of each species (Gupta *et al*., 2022; Zabala *et al*., 2018).

The possibility that the BMP-15 levels from the COCs group exposed to 30% EG + 1 M sucrose (P2) differed from the P1 group and the control group is due to the cryoprotectant interacting with the cumulus cells first. It is estimated that EG concentrations above 20% can cause most cumulus cells to die and become damaged, so they are rarely vitrified with oocytes. One way to avoid cumulus cell death is to reduce the concentration of one type of cryoprotectant and then mix it with another type of cryoprotectant such as EG-DMSO or EG-PROH (López *et al*., 2021; Safitri *et al*., 2023). The mechanism of EG or DMSO as harmful substances stimulated by the cumulus-oocyte complex, especially cumulus cells, causes the oocyte to activate the detoxification system from enzymes during the warming process. These enzymes include cytochrome p450, UDP-glucuronosyltransferase, sulfotransferase, acetyltransferase, and epoxide hydrolase to reduce the concentration of EG or DMSO into polar groups that can be accepted by the oocyte's intracellular fluid. This also activates the paracrine protection system, namely the secretion of BMP-6, BMP-7, and BMP-15 (Sanfins *et al*., 2018; Purnama *et al*., 2019). Meanwhile, IGF-1 did not differ significantly among the three groups of goat COCs, known to be unaffected by cryoprotectants, as stated correctly by Chen *et al*. (2017) and Rienzi *et al*. (2017). However, Yoshida *et al*. (2020) stated that IGF-1 regulates IGF-1-like signaling, producing HSP1A in cumulus cells transported to the oocyte to maintain COCs homeostasis and regulate BAX and HSP1 to prevent activation of apoptosis genes.

CONCLUSION

Commercial cryoprotectants demonstrated superior performance compared to the combination of EG and sucrose in the vitrification process of COCs. This superiority is evidenced by higher post-warming survival rates, as indicated by enhanced IGF-1 expression. Furthermore, commercial cryoprotectants exhibited greater efficacy in maintaining BMP-15 expression following the warming process. These findings suggest that commercial cryoprotectants may be more suitable for maintaining the integrity and developmental potential of COCs during vitrification and subsequent warming processes.

ACKNOWLEDGEMENTS

The authors would like to thank the Novice Research Scheme for Higher Education Excellence (PDUPT) Ministry of Education, Culture, Research, and Technology, Republic of Indonesia.

AUTHORS' CONTRIBUTIONS

WW: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing an original draft, Visualization, Supervision, Project administration, Funding acquisition. RR: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing an original draft, Visualization, Supervision, Project administration, Funding acquisition. TDL, ES, MM: Conceptualization, Methodology, Validation, Formal analysis, Investigation. ZS, VFH: Writing original draft, Visualization, Review and Editing.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

Ahuja, K. K., & Macklon, N. (2020). Vitrification and the demise of fresh treatment cycles in ART. *Reproductive Biomedicine Online*, 41(2), 217–224.

Andreis, A., Imazio, M., Casula, M., Avondo, S., & De Ferrari, G. M. (2021). Colchicine efficacy and safety for the treatment of cardiovascular diseases. *International Emerging Medicine*, 16(6), 1691–1700.

Awan, M., Buriak, I., Fleck, R., Fuller, B., Goltsev, A., Kerby, J., Lowdell, M., Mericka, P., Petrenko, A., Petrenko, Y., Rogulksa, O., Stolzing, A., & Stacey, G. N. (2020). Dimethyl Sulfoxide: A Central Player Since the Dawn of Cryobiology, is Efficacy Balanced by Toxicity?. *Regenerative Medicine*, 15(3), 1463–1491.

Azari, M., Kafi, M., Ebrahimi, B., Fatehi, R., & Jamalzadeh, M. (2017). Oocyte maturation, embryo development and gene expression following two different methods of bovine cumulus-oocyte complexes vitrification. *Veterinary Research Communications*, 41(1).

Bahari, B. S., Agoeshermadi, H., Fikri, F., Sardjito, T., Purnomo, A., Chhetri, S., Maslamama, S. T., & Purnama, M. T. E. (2023). Efficacy of intra-vulvo submucosal prostaglandins on estrous cycles in goats. *Indian Veterinary Journal*, 100(4), 24–27.

Best, B. P. (2015). Cryoprotectant Toxicity: Facts, Issues, and Questions. *Rejuvenation Research*, 18(5), 422–436.

Borges, A. B., de Queiroz, N. L., Santos, M., Oliveira, M., Silva, A., & Pereira, A. (2018). Combination of ethylene glycol with sucrose increases survival rate after vitrification of somatic tissue of collared peccaries (*Pecari tajacu* Linnaeus, 1758). *Pesquisa Veterinária Brasileira*, 38, 350–356.

Campbell, L. H., & Brockbank, K. G. M. (2022). Development of a Vitrification Preservation Process for Bioengineered Epithelial Constructs. *Cells*, 11(7), 1115.

Chang, C. C., Shapiro, D. B., Nagy, Z. P. (2022). The effects of vitrification on oocyte quality. *Biology Reproduction*, 106(2), 316–327.

Chen, P., Pan, Y., Cui, Y., Wen, Z., Liu, P., & He, H. (2017). Insulin-like growth factor I enhances the developmental competence of yak embryos by modulating aquaporin 3. *Reproduction Domestication Animals*, 52, 825–835.

Chen, Z. J., Li, M., Li, Y., Zhao, L. X., Tang, R., Sheng, Y., Gao, X., Chang, C. H., & Feng, H. L. (2014). Effects of sucrose concentration on the developmental potential of human frozen-thawed oocytes at different stages of maturity. *Human Reproduction*, 19(10), 2345–2349.

Dai, S., Hanxue, Z., Feng, Y., Wei, S., & Shenming, Z. (2022). Effects of IGF-1 on the Three-Dimensional Culture of Ovarian Preantral Follicles and Superovulation Rates in Mice. *Biology*, 11(6), 833.

Doyle, J. O., Richter, K. S., Lim, J., Stillman, R. J., Graham, J. R., & Tucker, M. J. (2016). Successful elective and medically indicated oocyte vitrification and warming for autologous in vitro fertilization, with predicted birth probabilities for fertility

preservation according to number of cryopreserved oocytes and age at retrieval. *Fertility and Sterility*, 105(2), 459–66.e2.

Edashige, K. (2016). The movement of water and cryoprotectants across the plasma membrane of mammalian oocytes and embryos and its relevance to vitrification. *The Journal of Reproduction and Development*, 62(4), 317–321.

Gupta, P. S. P., Kaushik, K., Johnson, P., Krishna, K., Nandi, S., Mondal, S., Nikhil Kumar Tej, J., Somoskoi, B., & Cseh, S. (2022). Effect of different vitrification protocols on post thaw viability and gene expression of ovine preantral follicles. *Theriogenology*, 178, 1–7.

Hanifah, N. F., Ratnani, H., Purnama, M. T. E., Restiadi, T. I., Agustono, B., & Prastiya, R. A. (2020). Effect of Glycerol Concentration in Tris Diluents on Spermatozoa Quality of Sapera Goats Before Freezing. *Jurnal Medik Veteriner*, 3(2), 154–159.

Hardy, K., Wright, C. S., Franks, S., & Winston, R. M. (2000). In vitro maturation of oocytes. *British Medical Bulletin*, 56(3), 588–602.

Herdis, M., Surachman, I. W. A., & Darmawan, A. (2019). The role of sucrose as extracellular cryoprotectant in maintaining the Garut rams' frozen semen quality. *AIP Conference Procedding*, 2120(1), 080019.

Juliawan, I. M. P., Suwana, F. P., Annas, J. Y., Akbar, M. F., & Widjiati, W. (2022) High Sucrose and Cholic Acid Diet Triggers PCOS-like Phenotype and Reduces Enterobacteriaceae Colonies in Female Wistar Rats. *Pathophysiology*, 29(3), 344–353.

Krause, A. R. T., Dias, F. C. F., Caunce, S. L., Adams, G. P., Mapletoft, R. J., & Singh, J. (2022). Predictors of the ovarian superstimulatory response and oocyte collection in prepubertal heifers. *Domestic Animal Endocrinology*, 81, 106729.

Llonch, S., Barragán, M., Nieto, P., Mallol, A., Elosua-Bayes, M., Lorden, P., Ruiz, S., Zambelli, F., Heyn, H., Vassena, R., & Payer, B. (2021). Single human oocyte transcriptome analysis reveals distinct maturation stage-dependent pathways impacted by age. *Aging Cell*, 20(5), e13360.

López, B., Ravassa, S., Moreno, M. U., José, G. S., Beaumont, J., González, A., & Díez, J. (2021). Diffuse myocardial fibrosis: mechanisms, diagnosis and therapeutic approaches. *Nature Reviews Cardiology*, 18(7), 479–498.

Mishra, A., Gupta, P. S. P., Sejian, V., Reddy, I. J., & Ravindra, J. P. (2016). Maturation timing and fetal bovine serum concentration for developmental potential of sheep oocytes in vitro. *Indian Journal of Experimental Biology*, 54(10), 630–633.

Nemer, A., Azab, A. N., Rimon, G., Lamprecht, S., & Ben-Menahem, D. (2018). Different roles of cAMP/PKA and PKC signaling in regulating progesterone and PGE2 levels in immortalized rat granulosa cell cultures. *Genetic Comparative Endocrinology*, 269, 88–95.

Novus Biologicals. (2021). Immunochemistry Handbook. USA. https://www.novusbio.com/support/handbooks-and-guides.

Prastiya, R. A., Samik, A., Purnama, M. T. E., & Saputro, A. L. (2019). Protective effect of mycotoxin binders on ovarian gestation mice exposed by zearalenone. *Indian Veterinary Journal*, 96(9), 44–46.

Purnama, M. T. E., Dewi, W. K., Prayoga, S. F., Triana, N. M., Aji, B. S. P., Fikri, F., & Hamid, I. S. (2019). Preslaughter stress in banyuwangi cattle during transport. *Indian Veterinary Journal*, 96(12), 50–52.

Rao, B. S., Mahesh, Y. U., Charan, K. V., Suman, K., Sekhar, N., & Shivaji, S. (2012). Effect of vitrification on meiotic maturation and expression of genes in immature goat cumulus oocyte complexes. *Cryobiology*, 64(3), 176–184.

Rienzi, L., Gracia, C., Maggiulli, R., LaBarbera, A. R., Kaser, D. J., Ubaldi, F. M., Vanderpoel, S., & Racowsky, C. (2017). Oocyte, embryo and blastocyst cryopreservation in ART: systematic review

and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Human Reproduction Update*, 23(2), 139–155.

Safitri, E., Purnobasuki, H., Purnama, M. T. E., & Chhetri, S. (2022). Effectiveness of forest honey (*Apis dorsata*) as therapy for ovarian failure causing malnutrition. *F1000Research*, 11, 512.

Safitri, E., Purnobasuki, H., Purnama, M. T. E., & Chhetri, S. (2023). Role of apoptotic inhibitors, viability, and differentiation in low oxygen tension of mesenchymal stem cells cultured in a rat model of ovarian failure. *F1000Research*, 12, 24.

Sanaei, B., Movaghar, B., Valojerdi, M. R., Ebrahimi, B., Bazrgar, M., Jafarpour, F., Nasr-Esfahani, M. H. (2018). An improved method for vitrification of in vitro matured ovine oocytes; beneficial effects of Ethylene Glycol Tetraacetic acid, an intracellular calcium chelator. *Cryobiology*, 84, 82–90.

Sanfins, A., Rodrigues, P., & Albertini, D. F. (2018). GDF-9 and BMP-15 direct the follicle symphony. *Journal of Assisted Reproduction and Genetics*, 35(10), 1741–1750.

Wang, S., Yang, H., Fu, Y., Teng, X., Wang, C., & Xu, W. (2022). The Key Role of Peroxisomes in Follicular Growth, Oocyte Maturation, Ovulation, and Steroid Biosynthesis. *Oxidation Medical Cell Longevity*, 2022, 7982344.

Whaley, D., Damyar, K., Witek, R. P., Mendoza, A., Alexander, M., & Lakey, J. R. (2021). Cryopreservation: An Overview of Principles and Cell-Specific Considerations. *Cell Transplantation*, 30, 963689721999617.

Widjiati, Desi, W, Wurlina, & Ngakan, M. R. W. (2011). The identification of Growth Differentiation Factor-9 from in vitro matured bovine oocytes by immunocytochemistry. *Journal of Veterinary Medicine*, 8(1).

Yoshida, T., & Delafontaine, P. (2020). Mechanisms of IGF-1-Mediated Regulation of Skeletal Muscle Hypertrophy and Atrophy. *Cells*, 9(9), 1970.

Zabala, A., Vazquez-Villoldo, N., Rissiek, B., Gejo, J., Martin, A., Palomino, A., Perez-Samartín, A., Pulagam, K. R., Lukowiak, M., Capetillo-Zarate, E., Llop, J., Magnus, T., Koch-Nolte, F., Rassendren, F., Matute, C., & Domercq, M. (2018). P2X4 receptor controls microglia activation and favors remyelination in autoimmune encephalitis. *EMBO Molecular Medicine*, 10(8), e8743.

Zhang, T., Xi, Q., Wang, D., Li, J., Wang, M., Li, D., Zhu, L., & Jin, L. (2019). Mitochondrial dysfunction and endoplasmic reticulum stress involved in oocyte aging: an analysis using single-cell RNA-sequencing of mouse oocytes. *Journal of Ovarian Research*, 12(1), 53.

***