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Effect of Heat Shock through MDA and 8-OHdG Levels of Post-Thawing Goat Oocytes

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ABSTRACT

The text explores crucial technologies for safeguarding genetic resources, particularly local goats in Indonesia, emphasizing methods to protect oocytes essential for in vitro maturation and assisted reproductive technologies (ARTs) or stocks. Vitrification is identified as the optimal choice for preserving oocyte stocks. The study investigates the impact of heat shock on thawed goat oocytes, focusing on Malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels. Comparing a commercial cryoprotectant (P1) with ethylene glycol 30% and 1M sucrose (P2), the results reveal a significant difference in MDA levels between P1 (0.081±0.023) and P2 (0.421±0.053). Additionally, a trend suggests no statistically significant difference in 8-OHdG levels between commercial media (7614.08±250.56) and EG 30% + 1M sucrose (7815.02±191.91). In conclusion, the study underscores the complexities associated with cryoprotectant concentration, confirming that ethylene glycol 30% with 1M sucrose can effectively vitrify goat oocytes with careful exposure during warming and adherence to vitrification protocols.

Key words: In vitro maturation, MDA, 8-OhdG, Oocyte, Vitrification, Food security

INTRODUCTION

The presence of local goats is under threat due to the adoption of Assisted Reproductive Technology (ART), such as Artificial Insemination (AI), and the importation of larger goat breeds like Boer goats from Australia. The prevalence of imported larger goats has marginalized the local goat population, exacerbated by the absence of a centralized recovery center for local goats. A significant challenge is the limited understanding of the genetic variations in local goats. Technological reinforcement is essential to protect genetic resources and conserve local goat populations. Preserving the pure genetics of these goats involves safeguarding not only the sperm but also the oocytes from the female goats. Oocytes play a crucial role in fertilization and successful pregnancies during both natural mating and in vitro fertilization treatments. However, the quality of oocytes can be negatively impacted by in vitro maturation processes (Martínez-Rodero et al. 2022). Once the oocytes mature, they can be utilized for in vitro fertilization, embryo transfer, or undergo vitrification. Vitrification is a complex process that includes exposing oocytes to increasing concentrations of cryoprotectants, both permeable and non-permeable, to prevent the formation of ice crystals inside and outside the cells before storing them in liquid nitrogen (Marques et al. 2018).

In the context of in vitro maturation technology, the objective is to generate mature oocytes as a source of gamete cells for embryo production in local goats. Cryoprotectants are crucial in the oocyte freezing process to shield embryos from drastic temperature changes, reaching as low as -196°C. When subjected to heat shock, the oocytes rely on endogenous antioxidants to counteract the release of free radicals triggered by the heat shock. If the endogenous antioxidants fail to adequately suppress this effect, it results in increased lipid peroxidation and the generation of reactive oxygen species (ROS), leading to elevated levels of Malondialdehyde (MDA) and heightened induction of 8-hydroxy-2-deoxyguanosine (8-OHdG). Consequently, this process hampers essential metabolic processes vital for oocyte maturation. Two reactive oxygen species (ROS) byproducts are malondialdehyde (MDA), originating from lipid peroxide breakdown, and eight-hydroxy-2'-deoxyguanosine (8-OHdG). MDA has served as an indicator of cellular peroxidative damage (Oral et al. 2006).

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On the other hand, 8-OHdG is a sensitive marker for DNA damage resulting from oxidative stress. This compound emerges from DNA damage caused by various factors like radiation, hydroxyl radicals, superoxide, or peroxynitrite (Seino et al. 2002).

The aim of this research is to demonstrate the impact of heat shock on the thawing process of frozen oocytes concerning MDA and 8-OHdG levels, as well as the post-thawing quality and morphology of the oocytes. This research is expected to improve the quality of oocytes as part of providing high-quality frozen embryos and addressing animal-derived food security.

MATERIALS AND METHODS

Ethical clearance

Ethical clearance for this research was obtained from the Animal Care and Use Committee, Faculty of Veterinary Medicine (ACUC) No. 1.KEH.051.03.2023 with title of research "Effect of Heat Shock Through MDA and 8-OHdG Levels of Post-Thawing Goat Oocytes" taken from Faculty of Veterinary Medicine, Universitas Airlangga.

Experimental site and layout

This research method consists oocytes from local goats obtained from slaughterhouse and processed in In Vitro Laboratory at Faculty of Veterinary Medicine, Universitas Airlangga. There are two groups: the treatment group (P) involves oocytes resulting from in vitro maturation that are freezing to vitrification method using commercial cryoprotectant (P1) and ethylene glycol with sucrose (P2) then thawed. The research stages include collecting goat oocytes, in vitro oocyte maturation, oocyte vitrification, oocyte thawing, macroscopic examination of oocyte morphology, and examining the levels of MDA and 8-OHdG using ELISA.

Medium preparation

The medium was formulated for oocyte collection, oocyte maturation, in vitro fertilization, and in vitro culture. Subsequently, the formulated medium is distributed in the form of droplets and placed in a 5% CO2 incubator at temperature of 38.5°C one day prior for oocyte collection.

Oocyte collection

Goat ovaries obtained from the slaughterhouse were transported to the laboratory in a flask containing 0.95% NaCl at a temperature of 37°C. Upon arrival at the laboratory, the ovaries were removed from the hanging device, thoroughly cleaned, and washed with 0.95% NaCl along with 100 µl gentamycin until completely clear. Oocyte collection was performed by aspiration using a syringe equipped with an 18G needle containing 1mL of MEM medium. Subsequently, the collected oocytes were placed in a sterile petri dish and examined under a microscope. Only oocytes surrounded by cumulus complexes with more than 3 layers were selected for in vitro maturation.

In vitro maturation

Oocytes enclosed by more than 3 layers of cumulus cells underwent a triple wash using MEM medium. Afterward, they are moved to the designated maturation media and placed in a 5% CO_2 incubator at 38.5°C for 20 hours, enabling the cumulus cells to expand. The maturity of the oocytes was assessed.

Vitrification

The vitrification procedure of mature oocytes was performed according to the method described by commercial vitrification kit's protocol for vitrification P1, oocytes were incubated in ES for 15min, VS1 for 30s, and VS2 for 10s, then placed in hemi-straw and plunged into liquid nitrogen (LN_2) then placed inside mini-straw and packaged in cassettes, finally placed inside LN_2 container. For vitrification P2, oocytes were incubated in EG30% for 15minutes and 1M sucrose for 40 seconds, then placed in hemi-straw and plugged into liquid nitrogen (LN_2) then placed inside mini-straw and packaged in cassettes then placed also inside LN_2 container.

Thawing treatment of vitrified oocytes

P1 samples thawed by using a commercial thawing kit started from a four-well tissue culture containing TS in the first well, DS in the second well, and WS1 with WS2 in the two last wells. The cassettes containing oocyte straws were taken out from LN2 and placed into TS for 1min, after that aspirated the oocyte into DS well for 3min. In the next step, the oocytes were aspirated into WS1 for 5min and WS2 for 1min. After thawing, the oocytes were placed inside culture media for recovery in a room maintained at 25°C. P2 samples have the same procedure as P1 thawing treatment with four wells containing 0.25M, 0.5M and 1M sucrose in two wells.

Evaluation of oocyte morphology

Thawed oocytes were subsequently observed using the Inverted Routine Microscope ECLIPSE Ts2 (Nikon, Japan) at magnifications set at 100, 200 and 400x. The level of maturation for each treatment was calculated and an evaluation of the quality of individual oocytes was conducted. The assessment of oocyte quality relied on the identification of Polar Body II.

Examination of MDA and 8-OhdG

Oocytes that have been placed in culture media were aspirated then transferred into Eppendorf tubes containing 1.0mL of lysis buffer and vortexed for 15min, then centrifuged at 3000 rpm for 15min to separate the supernatant from pellets. The supernatant obtained was then measured for MDA and 8-OHdG using the enzyme-linked immunosorbent assay (ELISA) method with Mouse Malondialdehyde, MDA assay kit Code SH0020 Brand: BT LAB and 8-OHdG (8-Hydroxydeoxyguanosine) ELISA Kit catalogue number E-EL-0028 Brand Elabscience.

Data analysis

The data collected were processed through the Statistical Program of Social Sciences (SPSS) version 23.0, employing the Kruskal-Wallis test. In case of notable difference ($P \leq 0.05$), additional examination was undertaken using the Mann-Whitney test.

RESULTS

Data on MDA levels and 8-OHdG were measured with ELISA method for 2 groups of Kacang goat oocytes. After being analyzed, the data are presented in Table 1.

Table 1: MDA levels and 8-OHdG levels from different groups oocytes without vitrified with vitrified using commercial CPA and EG30% + 1M Sucrose

Group	Average MDA Levels	Average 8-OHdG Levels
Commercial CPA (P1)	0.081±0.023[a]	7614.08±250.56[a]
EG 30% + 1M Sucrose (P2)	0.421±0.053[b]	7815.02±191.91[a]

*Different letters within each column indicate a significant difference (P≤0.05).

In accordance with cryoprotectant selection, mature oocytes were divided into two groups and subjected to distinct treatments. The first treatment involved the utilization of a commercial cryoprotectant referred to as P1 for vitrified oocytes, while the second treatment employed a solution comprising 30% ethylene glycol with 1M sucrose, denoted as P2, for the same vitrified oocytes. Analysis conducted using the SPSS program indicated no statistically significant difference (P≥0.05) in 8-OHdG levels between the P1 and P2 groups, although a significant difference (P≤0.05) in malondialdehyde (MDA) levels was observed between the two groups. The results of the MDA levels presented in P1 (0.081±0.023) were lower than P2 (0.421±0.053), which could show that commercial cryoprotectants are able to withstand the increases in ROS. Along with 8-OHdG levels, P1 with 7614.08±250.56 and P2 with 7815.02±191.91 showed no significant difference.

Aside from Table 1 results, we also presented the pictures for the morphology of oocytes that we took before to proceed to in vitro maturation in 22 hours, as well as pictures of thawed oocytes. Fig. 1 shows the IVM oocytes and Fig. 2 shows the thawed oocytes.

DISCUSSION

From the results, which showed the oocytes using commercial cryoprotectants (P1) and the oocytes using EG30% and 1M sucrose, we figured that there is a lack of differentiation in oocyte treatment outcomes between the two cryoprotectants, particularly regarding their effectiveness in safeguarding oocyte competence against increased reactive oxygen species (ROS) (Fernandez et al. 2022), proved from MDA levels at P1=0.081±0.023 and P2=0.421±0.053 while 8-OHdG have no different results. We basically aim for cryoprotectants as we know there are various cryoprotectants like ethylene glycol, propylene glycol, sucrose, which have been used in vitrification protocols over the years (Vajta and Nagy 2006). However, potential damage may still occur due to icy crystal formation in both intra- and extracellular environments. Cryopreserving oocytes face challenges, with attempts to enhance vitrification utilizing higher cryoprotectant concentrations for minimizing ice formation (Mandawala et al. 2016). Oocytes are susceptible to damage at different levels, including the zona pellucida, plasma membrane, meiotic spindle, cytoskeleton, and other structures. Research across species like cows, sheep, and mice has provided diverse but correlated results. For instance, examining germinal vesicle and MII goat oocytes after warming revealed significant differences in normal morphology and cleavage, with MII oocytes displaying greater tolerance to vitrification compared to immature oocytes (Quan et al. 2014).

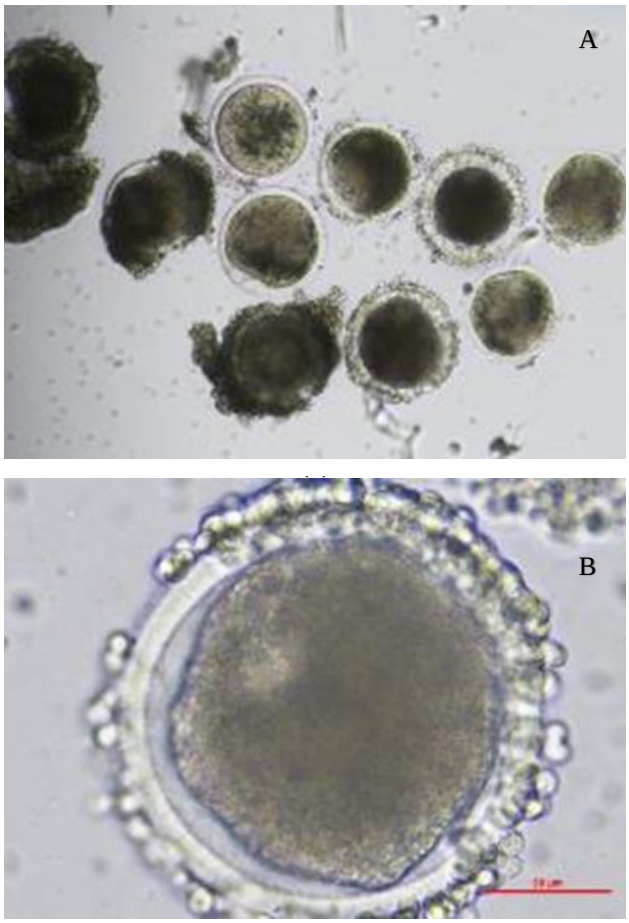

Fig. 1: The mature oocytes were collected and proceeded to in vitro maturation about 22 hours. Pictures from left to right: (A) mature oocytes with cumulus cells present in 100x display and (B) the mature oocyte with cumulus cells in 400x display.

Numerous studies have concurred that the key factor influencing oocyte competence vitrification and warming, particularly following in vitro maturation, is the crucial role played by cumulus cells in facilitating oocyte development. However, it has been observed that the layers of cumulus cells surrounding the oocyte may impede the exchange of water and cryoprotectants during the vitrification and warming process (Kasman et al. 2020). Another set of findings from various researchers indicates that oocytes vitrified without the surrounding cumulus cells, subsequent to in vitro maturation, exhibit increased viability but a simultaneous decrease in protein levels, fertility rates, and embryonic division rates. This variation is attributed to the use of different types and concentrations of vitrification and warming media (Bogliolo et al. 2007; Ortiz-Escribano et al. 2016). Consequently, additional studies propose that the observed effects of vitrification and warming on oocytes may result in disruption and damage to connexins, which are membrane channels facilitating communication between cells. This damage inhibits molecular exchange, leading to a lack of communication signals between oocytes and cumulus cells (Snoeck et al. 2018).

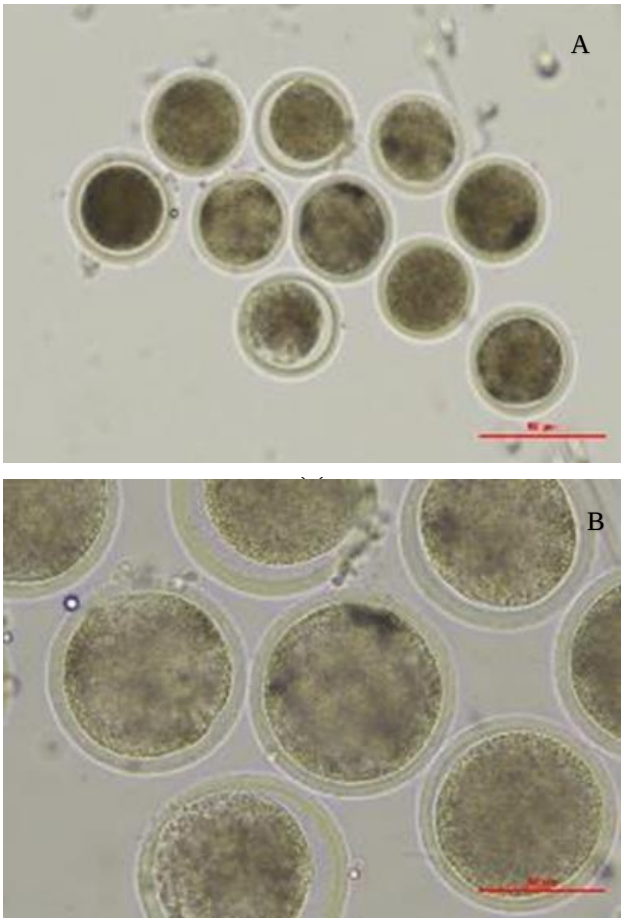

Fig. 2: The thawed oocytes were collected after seven days vitrificated inside N_2 container. From left to right: (A) the thawed oocytes present in 100x display and (B) the thawed oocytes present in 400x.

The vitrification process during oocyte warming is associated with the generation of reactive oxygen species (ROS), leading to elevated levels of lipid peroxidation, total antioxidant capacity and superoxide dismutase activity. This increase strongly correlates with oocyte fertilization and pregnancy rates. To counteract elevated ROS levels, the body prompts the production of nonenzymatic antioxidants and enzymes, including vitamins A, C, D, GSH, CAT, SOD, GST, PRDX and TXN. However, during in vitro fertilization (IVF), a decrease in GST, glutathione reductase, and GPX activity in follicular fluid can result in increased nitric oxide and the generation of lipid peroxidation byproducts such as malondialdehyde (MDA) and 4-HNE (Nuñez-Calonge et al. 2016).

The observed significant difference between P1 (0.081±0.023) and P2 (0.421±0.053) implies that the formation of malondialdehyde (MDA) through the Superoxide Dismutase (SOD), Glutathione Peroxidase (GPX) and Catalase (CAT) pathways is initiated by polyunsaturated fatty acids (PUFAs). PUFAs, vital components of cellular membranes, induce lipid peroxidation, compromising cell integrity and leading to cell degeneration. In the primary defense against reactive oxygen species (ROS), SOD catalyzes the dismutation of superoxide radicals (O_2) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), while CAT and GPX facilitate the breakdown of H_2O_2 into water (H_2O) and oxygen (O_2) (Agarwal et al. 2014). However, internal cellular processes may hinder antioxidant defense mechanisms, resulting in reduced defense capabilities, as evidenced by the presence of MDA (Agarwal et al. 2005). This reduction triggers an increase in oxygen (O_2) production, subsequently promoting lipid peroxidation.

During oocyte vitrification, a significant surge in MDA levels is expected, accompanied by a decline in antioxidant activities (SOD, CAT, and GPX) (Liu and Ling 2010; Kashka et al. 2016). Vitrification hinders SOD activity by directly affecting mitochondria (Salehnia et al. 2013). The reduction in SOD activity, along with elevated MDA levels, may indicate tissue injury due to the accumulation of O_2 radicals, typically eliminated by CAT (Agarwal et al. 2014). Despite CAT's collaboration with SOD to neutralize H_2O_2, diminished CAT activity in vitrified oocytes may signify cellular disorientation (Halliwell and Whiteman 2004; Halliwell 2014).

Analyzing the 8-OHdG results from thawed oocytes, we found P1 (7614.08±250.56) and P2 (7815.02±191.91) did not exhibit a significant difference, suggesting that the compared cryoprotectants did not induce a significant variation. However, this implies that oocytes undergo oxidative stress during the vitrification-to-warming process, leading to an increase in 8-OHdG levels. Several factors during the research, such as light exposure, oocyte handling, physicochemical parameters, and oxygen concentration, may have impacted oocyte physiology (Agarwal et al. 2014; Rocha-Frigoni et al. 2016). The hydroxyl radical (HO^-) interacts with DNA nucleobases, particularly guanine, resulting in the production of C8-hydroxyguanine (8-OHGua) or when interacting with its nucleoside (guanosine), forming deoxyguanosine (8-hydroxy-2-deoxyguanosine). ROS attack the 8th carbon atom of guanine in DNA, leading to the creation of 8-OHdG, an oxidized derivative of deoxyguanosine (Mukheef et al. 2022; Al-Anshori et al. 2023).

The outcomes derived from the measurement of Malondialdehyde (MDA) levels and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in two groups of vitrified oocytes prompt an inference on the cryoprotectant's capacity to withstand osmotic changes during sudden extreme temperature fluctuations in the vitrification process. Extensive research has been conducted to identify liquids capable of optimizing cellular dehydration in vitrification media, notably ethylene glycol, dimethyl sulfoxide (DMSO) and propylene glycol. Our comparative analysis focuses on ethylene glycol combined with sucrose versus a commercial cryoprotectant. In essence, our aim is to enhance the economical and practical aspects of the technique for implementation in various laboratory settings (Ting et al. 2013; Borges et al. 2018).

The selection of 30% ethylene glycol (EG) with 1M sucrose is supported by Wahjuningsih et al. (2010), who demonstrated its successful application in bovine oocytes with a survival rate of approximately 89%. Additionally, the vitrification of caprine ovarian tissue using 0.25M sucrose and 10% fetal bovine serum (FBS) with EG has proven to be the most efficient method (Carvalho et al. 2011). The EG-sucrose combination has exhibited superior tissue survival rates, especially in caprine preantral follicles, providing satisfactory results in terms of volumetric ratio and influencing solution properties. This mixture, followed by washes in a sucrose-containing medium, has

demonstrated optimal outcomes, reducing EG toxicity (Orief and Schultze-Mosgau 2005; Santos et al. 2007).

Ethylene glycol, functioning as a permeable cryoprotectant, is characterized by small, low-weight molecules capable of entering cells and binding with water molecules. This property limits the intracellular and extracellular water volume, thereby safeguarding intracellular organelles (Prentice and Anzar 2011). Consequently, EG is preferred for vitrification due to its rapid diffusion into cells and low toxicity (Orief and Schultze-Mosgau 2005). However, even with low toxicity, EG may induce a gradual increase in oxidative stress, potentially causing cellular damage (Bautista and Kanagawa 1998).

The incorporation of a permeable cryoprotectant in lower individual concentrations combined with a non-permeable agent serves to mitigate the toxicity associated with a specific cryoprotectant. Non-permeable cryoprotectants, such as disaccharides (e.g., sucrose), do not penetrate membranes but contribute to increased osmolality, minimizing intracellular ice formation, concentrating cytoplasmic macromolecules, and facilitating the intracellular vitrification process. Additionally, they prevent over-swelling of the oocyte (Rall 1987; Sieme et al. 2016; Fernandez et al. 2022). The volume ratio analysis suggests that EG, in combination with or without sucrose, is an optimal cryoprotectant, enhancing cell permeability and reducing osmotic changes during cooling or warming (Agca et al. 2005).

Conclusion

In conclusion, the utilization of a cryoprotective mixture consisting of 30% ethylene glycol (EG) and 1 M sucrose emerges as a viable option for the vitrification of goat oocytes. The assessment of cellular damage, as indicated by levels of Malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG), demonstrates comparable outcomes to those observed in vitrified oocytes employing a commercial cryoprotectant. This underscores the potential efficacy and suitability of the EG 30% and 1 M sucrose combination, positioning it as a promising alternative for cryopreservation in the context of goat oocyte vitrification.

Authors contribution

Widjiati: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing original draft, Visualization, Supervision, Project administration, Funding acquisition. Eka Pramytha Hestianah: Methodology, Validation, Investigation, Resources. Epy Muhammad Luqman: Methodology, Validation, Investigation, Resources. Sultan Fadhilla Taqwa, Zahra Shabira, Riski Lesta Mega, Jemy Caesar: Writing original draft, Visualization, Writing, Review and Editing.

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