The effect of glutathione antioxidant addition in maturation medium on the morphology of Garut sheep (Ovis aries) oocytes after vitrification

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Abstract. The study regarding the addition of glutathione antioxidants in the maturation medium in Garut sheep oocytes has been done. The purpose of this study was to evaluate the addition of glutathione antioxidants on oocyte morphology at post-vitrification. A total of 129 oocytes were matured in vitro using TCM-199 medium combined with glutathione antioxidants with a concentration of 0 mM (KK); 0.5 mM (KP1); 1 mM (KP2); and 1.5 mM (KP3). Oocytes were then vitrified using a combination of 15 % ethylene glycol and 15 % dimethyl sulfoxide as cryoprotectants. Oocyte evaluation was carried out after 7 days of storage in liquid nitrogen, including oocyte maturity and oocyte morphology. The percentages of mature oocytes are 53.13 % (0 mM ), 55.88 % (0.5 mM ), 59.38 % (1 mM ), and 67.74 % (1.5 mM ) and the percentages of normal oocytes are 56.25 % (0 mM ), 64.71 % (0.5 mM ), 71.88 % (1 mM ) and 87.10 % (1.5 mM ). The results of ANOVA tests showed that the data were not significantly different between groups (P > 0.05), but the percentage tends to increase with the increase of antioxidants concentration. The addition of glutathione antioxidants in the maturation medium seems to help maintain oocyte morphology until post-vitrification, although not significant.

Keywords: Glutathione, IVM, morphology, sheep, vitrification

1. Introduction

Garut sheep (Ovis aries) is an Indonesian sheep produced by crossing three different sheep; merino sheep, kaapstad sheep and local sheep [1-3]. Based on the Decree of the Minister of Agriculture No.2914/Kpts/OT.140/6/2011, Garut sheep is a wealth of genetic resources for local Indonesian cattle that need to be preserved. Conservation of genetic resources can be done ex situ by applying cryopreservation techniques in oocytes, sperm, or embryos [4, 5].

Cryopreservation is a technique used to store cells or tissues for a long time at very low temperatures (-196 °C) to reduce the rate of metabolism in cells so they can be used whenever needed [6-8]. Cryopreservation techniques can be carried out by two different methods; slow rate freezing and vitrification (fast freezing). Vitrification is a cell freezing method that applies rapid freezing rates by exposing cells directly into liquid nitrogen using high cryoprotectant concentrations [5, 7, 9]. The vitrification method is known to have several advantages over the slow freezing method, which includes
not applying gradual drop in temperature, avoid ice crystals formation, can form glass-like structures, and can be directly carried out in the field because they do not require special equipment [6, 10, 11].

Before vitrification, oocytes need to be matured in vitro. During the maturation process, the excessive Reactive Oxygen Species (ROS) can interfere the metabolism process, lead to oxidative stress and cause damage to cells [12, 13]. ROS concentrations in cells can be suppressed by antioxidants which can turn ROS into less reactive molecules by donating their electrons to ROS and neutralize it [14, 15]. One of the commonly antioxidants used is glutathione. Glutathione is a thiol tripeptide compound consisting of three amino acids; glycine, cysteine and glutamic acid. Glutathione is a natural endogenous antioxidant that can be synthesized by mammalian cells in the cytosol [16]. The thiol group (SH) in glutathione has an important role in maintaining redox conditions in cells through the process of oxidation and detoxification of dangerous compounds such as free radicals. The thiol (SH) group can be reversibly oxidized and reduced [14, 17].

The purpose of this study was to evaluate the addition of glutathione antioxidants with a concentration of 0.5 mM, 1 mM and 1.5 mM in the maturation medium on the quality of Garut sheep oocytes post-vitrification.

2. Materials and method

2.1. Ovaries and oocyte collection

Garut sheep ovaries were transported from slaughterhouse in physiological solutions Ringer Lactate (RL) containing gentamycin (40 mg/mL) at 37 °C. Ovaries were then washed twice in Ringer Lactate (RL) containing gentamycin. Oocytes in the ovary were collected using a slicing method in the dish containing BSA (3 mg/mL), MgCl\(_2\), CaCl\(_2\), and gentamycin.

2.2. In vitro oocytes maturation

A total of 129 oocytes with one or more layers of cumulus and finely granulated cytoplasm were selected and washed twice in maturation medium, then randomly distributed among the treatment groups. Oocytes were matured using Tissue Culture Medium (TCM-199) containing Bovine Serum Albumin (BSA) FAF (5 mg/mL), Follicle Stimulating Hormone (FSH) (10 μg/mL), human Chorionic Gonadotrophin (hCG) (10 μg/mL), estradiol (1 μg/mL), sodium pyruvate (0.25 mM), and gentamycin with the addition of 0 mM (Control), 0.5 mM (KP1), 1 mM (KP2) and 1.5 mM (KP3) GSH. Oocytes were matured in spot containing 50 μL of maturation medium with different GSH concentrations for 5–10 oocytes and then covered with mineral oil. Oocytes were incubated for 24 hours at 38.5 °C and 5 % CO\(_2\).

2.3. Oocytes vitrification

Oocytes vitrification were done using a closed pulled straw system with vitrification medium containing 3 % BSA + 15 % DMSO + 15 % EG + 0.5 M sucrose. Oocytes were equilibrated in equilibration medium containing 3 % BSA + 10 % EG for 3–5 minutes. After equilibration the oocytes were loaded into a 0.25 mL straw. Straws were filled with sucrose followed by air space, then sucrose, air space, vitrification medium, air space, then vitrification medium containing oocyte, followed by air space, then sucrose. The open end of straws was then sealed and pre-cooled in liquid nitrogen vapors for 10 seconds. The straws were stored in liquid nitrogen for 7 days [18].

2.4. Thawing

Oocytes were thawed after 7 days of storage in liquid nitrogen. Straws from liquid nitrogen was put at room temperature for 6 seconds and then immersed in a water bath at 37 °C for 30 seconds. Straws were then flicked slowly so that the solution inside the straw was mixed. The straw contents were removed onto 200 μL of medium thawing containing 3 % BSA and 0.25 M sucrose. Oocytes were subsequently
denuded with hyaluronidase and then transferred to an adaptation medium containing 3 % BSA and gentamycin.

2.5. Oocytes quality evaluation

Evaluation of oocyte maturity was seen based on the presence of a first polar body. Oocytes with a first polar body were mature. The evaluation of oocyte morphology was based on oocyte shape, zona pellucida conditions, and oocyte cytoplasmic conditions.

2.6. Statistical analysis

The data were analyzed statistically using Statistical Product and Service Solution (SPSS) software version 25.0. The normality of the data was tested using the Shapiro-Wilk test and the homogeneity of the data was tested using the Levene test. The significant differences between treatment groups were tested using a one-way ANOVA.

3. Results and discussion

The total number of Garut sheep oocytes used in the study were 129 oocytes. The percentages of mature oocytes are 0 mM (53.13 %), 0.5 mM (55.88 %), 1 mM (59.38 %) and 1.5 mM (67.74 %) (table 1). The results of the one-way ANOVA statistical test showed the data did not significantly different between groups.

The results of the one-way ANOVA statistical test showed that the addition of GSH in the maturation medium did not have a significant effect on the number of mature oocytes. Similar results were also reported by Hasbi et al. using a concentration of 0 mM, 0.25 mM, 0.5 mM and 1 mM in sheep oocytes [19]. de Matos et al. stated that GSH is synthesized during the oocyte maturation process [20], so that the adding glutathione antioxidants does not give a significant difference to the number of mature oocytes [19].

Although the statistical test showed the data were not significantly different between treatment groups, the number of mature oocytes showed a percentage that tended to increase with the increase in the concentration of GSH given. This may be due to the addition of glutathione antioxidants which can increase the concentration of intracellular GSH. Mayor et al. reported that prepubertal goat oocytes matured with the addition of 1 mM GSH had significantly higher intracellular GSH concentrations (3.23 pmol/oocytes) compared to the control group (1.70 pmol/oocytes) and immature oocytes (1.21 pmol/oocytes) [21].

Glutathione can act as an antioxidant against ROS that formed during the maturation process. The thiol group (SH) in glutathione has an important role in maintaining redox conditions in cells through the process of oxidizing and detoxifying dangerous compounds. Reduced form glutathione (GSH) will donate hydrogen atoms in the thiol group (SH) to free radicals [14, 17]. The ability of antioxidant glutathione against ROS can protect cells from oxidative stress which can damage cells [22].

Table 1. Effect of addition of glutathione on oocytes maturity and morphology.

| Glutathione (mM) | Oocytes (n) | Mature oocytes after in vitro maturation (%) (n ± SD) | Oocytes with normal morphology (%) (n ± SD) |
|----------------|-------------|--------------------------------------------------|-------------------------------------------|
| 0 mM           | 32          | 17 ± 2.87 (53.13 %)*                              | 18 ± 2.89 (56.25 %)*                      |
| 0.5 mM         | 34          | 19 ± 1.71 (55.88 %)*                              | 22 ± 1.29 (64.71 %)*                      |
| 1 mM           | 32          | 19 ± 2.63 (59.38 %)*                              | 23 ± 4.11 (71.88 %)*                      |
| 1.5 mM         | 31          | 21 ± 0.96 (67.74 %)*                              | 27 ± 2.01 (87.10 %)*                      |

The same letter (a and b) in the same column indicate no significant differences among the different groups (P > 0.05).
Figure 1. Morphological abnormalities in oocytes post-vitrification. (a) Oocyte changes in shape, (b) Fracture on zona pellucida, (c) Cytoplasm not intact and (d) Cytoplasm fragmented. Scale bar 20 μm.

The percentage of oocytes with normal morphology post-vitrification are 56.25 % (0 mM); 64.71 % (0.5 mM), 71.88 % (1 mM) and 87.10 % (1.5 mM) (table 1). One-way ANOVA test (P > 0.05) showed the data did not significantly differ between treatment groups. Oocytes are categorized as normal when oocytes have a round shape, intact zona pellucida, intact and homogeneous cytoplasm. Oocytes are categorized as abnormal, i.e. oocytes have a non-round shape, fracture on zona pellucida, cytoplasm not intact and not homogeneous or fragmented [23, 24]. Figure 1 shows abnormal oocyte morphology.

The result of one-way ANOVA test indicating the addition of GSH to the maturation medium did not have a significant effect on the post-vitrification normal oocyte morphology. However, the percentage of normal oocytes tend to increase as the GSH concentration increased. It is believed that the addition of GSH in the maturation medium tends to increase the number of mature oocytes. Mature oocytes are known to have more stable plasma membrane than immature oocytes, so they can be more resistant to the influence of the vitrification process [25, 26]. Quan et al. reported that goat oocytes that were vitrified at MII stage (80.91 %) had a higher normal oocyte than oocytes that were vitrified at GV stage (60.37 %) [26].

4. Conclusion
The addition of glutathione antioxidants to the maturation medium showed a tendency to increase the percentage of mature oocytes and normal oocytes as the glutathione concentration increased. This showed the possibility that all the concentrations of glutathione antioxidants tested could maintain oocyte quality until post-vitrification.

Acknowledgments
This study was supported by Department of Biology Faculty of Mathematics and Natural Sciences Universitas Indonesia and Research Centre for Biotechnology, Indonesian Institute of Sciences.

References
[1] Inounu I, Subandiyo, Tiesnamurti B, Hidajati N and Nafiu L O 2005 JITV 10 17-26
[2] Mansjoer S S, Kertanugraha T and Sumantri C 2007 Media Peternakan 30 129-38
[3] Heriyadi D, Sarwesti A and Nurachma S 2012 Bionatura-Jurnal Ilmu-ilmu Hayati dan Fisik 14 101-6
[4] Andrabi S M H and Maxwell W M C 2007 Animal Reprod. Sci. 99 223-43
[5] Prentice J R and Azzar M 2011 Vet. Med. Int. 2011 146405
[6] Pamungkas F A 2010 Wartazoa 20 112-8
[7] Barun S 2015 *Beats Nat. Sci.* 2 1-6
[8] Mandawala A A, Harvey S C, Roy T K and Fowler K E 2016 *Theriogenology* 86 1637-44
[9] Jang T H et al. 2017 *Integr. Med. Res.* 6 12-8
[10] Amorim C A, Curaba M, Langendonckt A V, Dolmans M-M and Donnez J 2011 *Reprod. BioMed. Online* 23 160-86
[11] Quan G, Wu G and Hong Q 2017 *Biopreserv. Biobank.* 15 1-13
[12] Gale S L, Burritt D J, Tervit H R, Adams S L and McGowan L T 2014 *Theriogenology* 82 779-89
[13] Succu S et al. 2014 *Theriogenology* 81 1058-66
[14] Lobo V, Patil A, Phatak A and Chandra N 2010 *Pharmacogn. Rev.* 4 118-26
[15] Zhang W, Yi K, Chen C, Hou X and Zhou X 2012 *Animal Reprod. Sci.* 132 123-8
[16] Yuniastuti A 2016 *Dasar Molekuler Glutation dan Perannya sebagai Antioksidan* (Semarang: FMIPA Press, Universitas Negeri Semarang)
[17] Luberda Z 2005 *Reprod. Biol.* 5 5-17
[18] Andini D S 2017 *Pengaruh Berbagai Konsentrasi Etilen Glikol dan Dimetil Sulfoksida dalam Vitrifikasi terhadap Perkembangan Kultur Blastokista Awal Mencit (Mus musculus L.) Galur DDY Hasil Laser Assisted Hatching* Bachelor Thesis (Depok: Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia)
[19] Hasbi, Gustina S, Setiadi M A and Supriatna I 2012 *Jurnal Veteriner* 13 445-52
[20] de Matos D G, Furnus C C and Moses D F 1997 *Biol. Reprod.* 57 1420-5
[21] Mayor P, López-Béjar M, Rodríguez-Gonzáles E and Paramio M T 2001 *Zygote* 9 323-30
[22] de Matos D G, Gasparini B, Pasqualini S R and Thompson J G 2002 *Theriogenology* 57 1443-51
[23] Purohit G N, Meena H and Solanki K 2012 *J. Reprod. Infertil.* 13 53-9
[24] Kumar S, Gautam S K and Gahlawat S K 2013 *Int. J. Animal Biotechnol.* 3 14-8
[25] Chaves D F, de Souza-Fabjan J M G, Mermillod P and Freitas V J de F 2014 *R. Bras. Ci. Vet.* 21 69-75
[26] Quan G B et al. 2014 *Small Ruminant Res.* 116 32-6