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THE ROLE OF GSH SUPPLEMENTS IN MATURATION MEDIA IN IMPROVING THE BALI CATTLE OOCYTE FERTILITY BY IN VITRO TECHNIQUE

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Abstract
In the in vitro oocyte culture process, oxidative stress often occurs, which produces free radicals, so it was necessary to add the antioxidant glutathione (GSH). This research aimed to determine the fertility level of Bali cattle oocytes by adding glutathione (GSH) to the medium maturation. The study was conducted at the In Vitro Embryo Production Laboratory, Institute for Research and Community Service (LPPM) Building, Hasanuddin University in March-May 2023. The study consisted of 4 treatments with the addition of GSH (0 mM, 0.5 mM, 1 mM, and 1.5 mM) with 6 repetitions. The mature oocytes were then subjected to in vitro fertilization. The results showed that the highest fertilization rate (2 PN/Pronucleus) was significantly higher (P <0.05) in the treatment with the addition of 1-1.5 mM GSH to medium maturation, compared to 0.5 mM and without GSH addition, whereas fragmented oocytes (0 PN), (1PN) and (>2 PN) were not significantly different. This study concluded that adding 1.5 mM glutathione (GSH) to the maturation medium showed the best results, namely 72.26 % fertilized oocytes.
Keywords: Bali cattle oocytes, glutathione, in vitro fertilization.

PERANAN SUPLEMEN GSH PADA MEDIA MATURASI UNTUK MENINGKATKAN FERTILITAS OOSIT SAPI BALI SECARA IN VITRO

Abstrak
Pada proses kultur oosit in vitro sering terjadi stress oksidatif yang menghasilkan radikal bebas sehingga perlu ditambahkan antioksidan glutathione (GSH). Oleh sebab itu, research ini dilakukan untuk menganalisis tingkat fertilitas oosit sapi Bali dengan penambahan glutathione (GSH) pada media maturasi. Penelitian dilaksanakan di Laboratorium Produksi Embrio in Vitro, Gedung Lembaga Penelitian dan Pengabdian Masyarakat (LPPM), Universitas Hasanuddin pada bulan Maret-Mei 2023. Penelitian terdiri dari 4 perlakuan penambahan GSH (0 mM, 0,5 mM, 1 mM, dan 1,5 mM) dengan 6 kali ulangan. Oosit hasil maturasi selanjutnya dilakukan fertilisasi in vitro. Hasil penelitian menunjukkan bahwa tingkat fertilisasi tertinggi (2 PN/Pronucleus) nyata lebih tinggi (P < 0,05) pada penambahan GSH 1-1,5 mM pada medium maturas, dibanding 0,5 mM dan tanpa GSH, sedangkan untuk oosit terfragmentasi (0 PN), (1PN) dan (>2 PN) tidak berbeda nyata. Kesimpulan penelitian yaitu bahwa penambahan 1,5 mM glutathione (GSH) pada medium maturasi menunjukkan hasil terbaik yaitu 72.26% oosit yang terfertilisasi.
Kata kunci: oosit sapi bali , glutathione, , fertlisasi in vitro

INTRODUCTION

In vitro oocyte maturation (IVM) and in vitro fertilization (IVF) procedures require a substantial number of oocytes to carry on the process. Oocytes are obtained from the ovaries of female animals, and in the case of cattle, these ovaries can be conveniently and inexpensively sourced from slaughterhouses (RPH) (Ikhwan et al., 2016). Following retrieval, the oocytes are cultured in vitro within a 5% CO2 incubator, a process known as IVM.

The success of the Embryo Transfer Program relies on the quality of mature oocytes achieved through In Vitro Maturation (IVM), a critical step in the IVF process (Harissatria, 2012). The overall success of IVM is determined by the quality of immature oocytes,

the maturation medium, and the substances added to the medium (Wattimena, 2011). To enhance the success rate of IVM, Adifa et al. (2010) suggest adding gonadotropin hormones, serum, and antioxidants, such as vitamin E, Insulin Transferrin Selenium (ITS), and glutathione (GSH) to the medium.

Adding GSH antioxidants plays a significant role in the oocyte maturation process. GSH serves as an oocyte's protector against toxic ROS (reactive oxygen species) activity (Triwulanningsih et al., 2002). As an antioxidant agent, GSH will protect and maintain the cell nucleus and DNA and RNA chains in it against free radical particles' attack, preventing it from decomposing. GSH also repels unneeded substances from being excreted in the urine and the bile (Sugiyanta, 2008).

Previous research showed that adding 0 mM, 2 mM, 5 mM, 0.5 mM, and 1 mM GSH to the medium showed a number of sheep oocytes that reached the metaphase II (MII) stage, respectively 79.71%, 79.07%, 80.95% and 84.13% (P>.05) (Hasbi et al., 2012). Adding GSH 1.5 mM in the maturation medium, after being fertilized *in vitro,* showed a very significant level of fertilized oocytes (P<0.01) with a value of 88.98% which was higher than the maturation with the GSH treatment of 0.5 mM (48.50%) and 1.0 mM (48.50%) (Harissatria & Hendri, 2016).

Bali cattle oocyte fertilization rate with the addition of GSH on the maturation media is highly determined by the quality of the cultured oocytes. The oocytes used were selected based on compact cytoplasmic homogeneity and cumulus oophorus (COC). This selection process ensured that the immature oocytes were uniform and had similar developmental potential. Higher-quality oocytes are more likely to respond positively to GSH treatment, leading to enhanced fertilization rates and potentially improved reproductive outcomes for Bali cattle.

After the maturation process, the oocytes will undergo an *in vitro fertilization* (IVF) process to produce quality frozen embryos in large quantities to meet the needs of the embryo transfer program. Therefore, this research aims to determine the fertility level of cultured Bali cattle oocytes with GSH supplements on maturation media.

MATERIALS AND METHODS

Oocyte collection

Cattle ovaries taken at the Tamangapa Slaughterhouse (RPH), Manggala, were put into a bottle containing transportation medium (9 grams of NaCl + distilled water) until reached a final volume of 1 liter, then 500 µl strep pen were added. It was brought to the *in vitro* embryo production laboratory of Hasanuddin University within a maximum of 4 hours. The ovaries were washed three times using 0.9% NaCl, followed by the slicing process to obtain oocytes in a petri dish. Furthermore, oocytes were selected using the Olympus SZ51 Japan microscope. Only grade A and B oocytes were collected to be matured. Grade A oocytes are surrounded by densely layered cumulus cells, have more than three layers and have homogeneous ooplasm. Meanwhile, grade B oocytes are surrounded by a dense cumulus layer, one to three layers with a homogeneous ooplasm, a rough appearance and a dark-colored zona pellucida (Budiyanto, 2013)**.** Grade A and B oocytes were followed by an *in vitro* maturation process.

In vitro maturation

Oocytes that have been selected (grade A and grade B) are ripened in maturing medium (1800 µI 1 solution TCM-199, 200 µI serum/FBS 10%, 20 µI FSH, 20 µI hCG, 4 µI gentamycin) in drop shape (80 µl/drop) for 4 drops. Then, GSH was added according to the determined treatment levels with 5-10 oocytes/drop, and closed with mineral oil (Sigma Chemical Co. St. Louis MO, USA). Maturation was carried out in an incubator with 5% CO_2 temperature 38,5 ºC for 24 hours (Hasbi et al., 2012)**.**

In vitro oocyte fertilization

Preparation: Tubes filled with Suzuki fertilization media (2000) (NaCl, KCL, NaH_2PO_4, $MgSO_4H_2O$, Sodium lactate 60% syrup, Hepes, $CaCl_22H_2O$, and Sodium pyruvate) were prepared. Frozen semen was thawed at 37°C for 20 seconds and put into the prepared tube. Semen was centrifuged for 5 minutes at 1800 rpm, discarding the supernatant and the remaining sediment. The fertilization medium was added and centrifuged again simultaneously and quickly. After centrifugation, the supernatant was removed again, and the semen sediment residue was

added to 650 µl fertilization medium to the precipitated semen, then homogenized and made 4 drops (80 µl/drop) in a fertilization petri dish. Mineral oil is added to cover the media so that the media and oocytes do not dry out and then equilibrated at 38.5 °C for 30 minutes.

Implementation of IVF: after the IVM process, some part of the cumulus-oocyte was released to ease sperm movement on entering the oocyte. It was then washed three times in a fertilization medium. After being washed, the oocytes were transferred to the fertilization medium, which had been equilibrated. Then it was incubated in incubator at 38.5 °C for 24 hours.

Fertilization status

After the fertilization process, the COC was cleaned from the oocyte using 0.25% hyaluronidase enzyme (Sigma, USA) by pipetting the oocytes until the oocytes become denuded. Denuded oocytes were transferred to an object glass that had been given vaseline and paraffin (9:1) in all four corners, then covered with a cover slip. The oocyte preparations were fixed with ethanol and acetate (3:1) for 3-5 days at room temperature. After the fixation, the preparations were put back into the absolute ethanol solution for one hour. Preparations that have been soaked, dried using a tissue, then stained with 2% acetoorcein for 5 minutes. Furthermore, the dyes on the preparations were cleaned using 25% acetic acid for observation under the Axio cam microscope.

Fertilized status: Oocytes with fragmentation conditions are oocytes that do not reach the developmental stage metaphase II (0 PN). The unfertilized oocyte has 1 PN, fertilized oocyte has 2 PN, whereas more than 2 PN are polyspermic oocytes.

Results Analysis

This study was experimental laboratory research with Completely Randomized Design (RAL) ANOVA consisting of 4 treatments and 6 replications with the following arrangement:

P0: Maturation medium without GSH 0.5 mM.
P1: Maturation medium with the addition of 0.5 mM GSH.
P2: Maturation medium with the addition of 1.0 mM GSH.
P3: Maturation medium with the addition of 1.5 mM GSH.

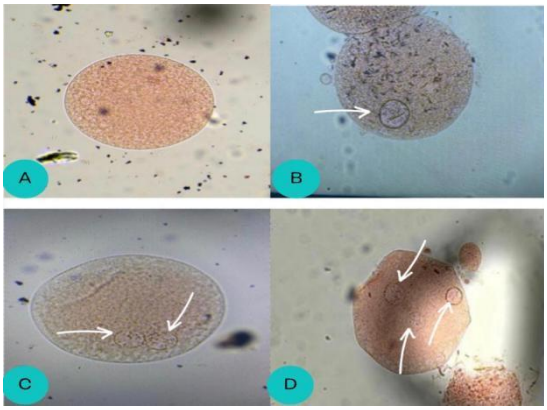
If there was a difference in the analysis of IVF results data, the Duncan test will proceed. Data was processed using the SPSS v16 program.

RESULTS AND DISCUSSION

Rate of *In Vitro Fertilization*

Fertilization is the process of meeting the nuclei of the ovum and spermatozoa cell nuclei to become a zygote. This process can occur inside the body (naturally or in vivo) and artificially occurring outside the body (in vitro). Elder and Dale (2011) stated that in vitro fertilization includes penetration of oocytes by spermatozoa, ovum activity, formation of female and male pronuclei and incorporation of maternal and paternal chromosomes to form the genome. The oocytes assessed were successfully fertilized oocytes which showed the presence of 2 pronuclei (2PN) consisting of male and female pronuclei. The success rate of oocyte fertilization in this study was seen from the discovery of polymorphonuclear, which is a male pronucleus as well as a female appearing on staining using 2% aceto orcein.

The results showed that the addition of GSH to the fertilization medium helped increase the formation of a normal 2PN pronucleus. The results of in vitro fertilization in Bali cattle oocytes showing the presence of a pronucleus (PN) are shown in Figure I.



Note: The arrows indicate the Pronucleus (PN); A. Fragmentation, B. 1 PN, C. 2 PN, and D. 3PN

Figure I. Overview of oocytes after *in vitro* fertilization.

Table I. IVF observation results Bali Cattle Oocytes

Treatment	N	Parameters (%)			
		0 PN	1 PN	2 PN	> 2PNs
P0	52	11(18.01±18.63) [a]	15(30.02±20.56) [a]	25(46.76±5.73) [a]	1(5.21±10.01) [a]
P1	48	2(4.16±10.21) [a]	18(37.03±7.33) [a]	28(58.79±6.13) [b]	0(.00±.00) [a]
P2	49	4(8.33±12.91) [a]	14(24.44±16.55) [a]	30(65.83±12.64) [bc]	1(1.38±3.40) [a]
P3	34	2(11.11±17.21) [a]	8(16.62±16.80) [a]	24(72.26±11.63) [c]	0(.00±.00)S [a]
Total	183	(10.40±15.01)	(27.03±16.82)	(60.92±13.14)	1(1.65±5.38)

Note: different letters in the same column indicate differences (P<0.05)
** P: Pronucleus

The research data after being analyzed showed that there was an effect of GSH administration in maturation medium on in vitro fertilization. Significant differences were seen in fertilized oocytes (2PN) between treatments (P<0.05), namely P0: 46.76%, P1: 58.79%, P2: 65.83%, and P3: 72.26%. Treatment using 1.5 mM (GSH) resulted in a higher percentage of fertilized oocytes, namely 72.26%. Another study showed that the results of adding 1 GSH to the maturation medium showed 86.9% of fertilized oocytes which were characterized by the formation of a normal pronucleus (2PN), while without the addition of GSH, it was 58.9% (Nugroho et al., 2017).

GSH concentration of 1.5 mM showed a better level of oocyte fertilization in Bali cattle. This is because glutathione sulfhydryl is a tripeptide thiol (y-glutamylcysteinylglycine), part of a non-protein sulphydryl which has an important function in the detoxification process as antioxidants maintaining/maintaining intracellular redox states and maintaining oxidative stress in the form of GSH to reduce as well as oxidizing forms (GSSG) (Luberda, 2005).

GSH (Glutathione) plays a crucial role mature in oocytes after fertilization by assisting in the formation of the male pronucleus (Zuelke et al., 2003). Within the oocyte cytoplasm, GSH naturally exists and is involved in various processes during pronucleus formation. It contributes to breaking the nuclear membrane disulfide bonds and initiating chromosome decondensation, facilitating the development of the male pronucleus (Maedomari et al., 2007). Glutathione plays an important role in cell protection, dividing, forming proliferation and maintaining T-lymphocyte cells which are the front line of defense against infection (Heisterkamp et al., 2008).

Spermatozoa that fail the acrosome reaction and capacitation are unable to enter the oocyte. Spermatozoa that failed in the condensation process in the oocyte cytoplasm causes fertilization failure (Crozet et al., 2000). Other factors also affect the results of in vitro fertilization, namely the production of Reactive oxygen species (ROS). Dead spermatozoa generate ROS, reduce membrane fluidity, and function of sperm and cause peroxidation of membrane lipids. High ROS can damage and disrupt the metabolism of spermatozoa in *In vitro* fertilization media. Kim et al. (2007) reported an increase in reactive oxygen species (ROS) levels under in vitro conditions when a 5% CO2 environment was used.

CONCLUSION

Addition of 1.5 mM glutathione (GSH) antioxidants in the maturation medium showed the best results obtained by the number of fertilized oocytes of 72.26 %. Oocyte fertility showed significant differences between different levels of GSH treatments (P<0.05).

SUGGESTION

More research on the additions of GSH antioxidants at a higher level of concentration is necessary to achieve higher optimal Bali cattle oocyte fertility.

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