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Rate Maturation, Fertilization and Hydrogen Peroxide Concentration of Bali Cow Oocytes with the Addition of Catalase and Hypotaurine

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ABSTRACT

This study was conducted to assess the effectiveness of catalase and hypotaurine on the rate of nuclear maturation, fertilization rate, and hydrogen peroxide concentration. This study was divided into three stages. The first stage is maturation media supplemented with catalase, the second stage is maturation media supplemented with hypotaurine, and the third stage is maturation and fertilization media supplemented with catalase, hypotaurine and a combination of both. This research employed compact cytoplasm and cumulus oocytes. After 24 hours in a 5% CO_2 incubator at 38.5°C, selected oocytes matured. After maturation, $1.5x10^6$ spermatozoa/mL fertilization medium was used in a 5% CO_2 incubator at 38.5°C for *in vitro* fertilization. Oocytes were tested for hydrogen peroxide, nuclear maturation, and fertilization after 24 hours. The study indicated that 150IU/mL catalase led to the highest percentage of oocytes reaching metaphase II (75.4±3.3), with no significant difference ($P>0.05$) from other treatments. Adding 150IU/mL catalase lowered H_2O_2 concentration (16.6) ($P<0.05$) compared to the control (28). Hypotaurine increased oocyte MII to 6 mM (86.9±1.8) after addition, considerably different from the control (64.3±3.0) ($P<0.05$). The H_2O_2 concentration in the 6 mM treatment (22.9) was significantly lower ($P<0.05$) than the control (36.0). The maximum fertilization rate (78.6±2.7) was significantly ($P<0.05$) higher than the control (67.2±1.3) in the 150IU/mL and 6mM treatment. Hydrogen peroxide levels in the combination treatment (33.7) were significantly lower ($P<0.05$) than the control (47.1). In conclusion, catalase and hypotaurine supplementation promotes metaphase II and fertilized oocytes.

Key words: Hypotaurine, Hydrogen peroxide, *In vitro* maturation, *In vitro* fertilization, Catalase

INTRODUCTION

In vitro embryo production (IVEP) has been advanced throughout the last two decades. *In vitro* maturation (IVM) and *in vitro* fertilization (IVF) did not enhance IVEP efficiency (Luciano et al. 2018). IVEP success depends on several aspects. Oxidative stress (OS) is a major issue (Khazaei and Aghaz 2017).

In vivo culture has 5 to 7% oxygen tension, whereas *in vitro* culture has 20%. This condition can disrupt mitochondrial function (Li et al. 2024), so these circumstances may boost ROS generation, including superoxide anions, lipid peroxides, and H_2O_2 (Morado et al. 2009; Gustina et al. 2019). Whereas under normal conditions, ROS play a role in helping the process of proliferation and differentiation (Valko et al. 2007; Sharifi-rad et al. 2020). As oocytes use oxygen for energy via mitochondrial oxidative phosphorylation, ROS generation rises *in vitro* (Amporn et al. 2023). Approximately 90% of cell ROS are produced from mitochondria (Al-Zubaidi et al. 2021; Zhou et al. 2022). In vivo, the antioxidant system to ward off free radicals is formed naturally (Vona et al. 2021), while in vitro conditions are different. Oxidative stress (OS) arises when reactive oxygen species generation surpasses the cell's antioxidant capacity (Gualtieri et al. 2021). Oxidative stress may induce chromosomal damage, lipid peroxidation, and mitochondrial dysfunction, which can lead to major health issues (Choi et al. 2007; Mihalas et al. 2017).

The equilibrium between reactive oxygen species and antioxidants affects sperm, oocyte and fertilization (Lapointe and Bilodeau 2003). Antioxidants that repair free radical damage protect oocytes from oxidative stress (Zarbakhsh 2021). According to the opinion of Rodríguez-Varela and Labarta (2020), using antioxidants can protect oocytes from oxidative stress. Antioxidants are compounds that can release their electron structure to break the chain of free radical compounds (Budani and Tiboni 2020).

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Antioxidants are enzymatic or non-enzymatic. Catalase reduces ROS and improves embryo development as an enzymatic antioxidant (Wang et al. 2013). Catalase breaks H_2O_2 into water and oxygen (Gupta et al. 2011). Hypotaurine, a non-enzymatic antioxidant, protects sperm viability and capacitation against peroxidation (Guerin et al. 1995). Hypotaurine also protects the embryo from oxidative stress (Guerin 2001). Suzuki and Yoshioka (2006) found that hypotaurine in culture medium reduces H_2O_2 and protects DNA. Therefore, catalase and hypotaurine can be used as antioxidants in maturation and fertilization media to increase maturation and fertilization rates and reduce hydrogen peroxide concentrations in *in vitro* bovine oocytes. This study was conducted to assess the effectiveness of catalase and hypotaurine on the rate of nucleus maturation, fertilization rate and hydrogen peroxide concentration.

MATERIALS AND METHODS

Ethical approval

The Animal Ethics Commission, Faculty of Veterinary Medicine, Udayana University, Denpasar, Indonesia, accepted this research, number B/143/UN14.2.9/PT.01.04/2024.

Research design

1. Stage 1. Addition of catalase to maturation media

The effect of catalase on oocyte maturation *in vitro* was examined. Oocytes were matured in M 199 media with four treatments, namely control, the addition of catalase 50IU/mL, 100IU/mL, and 150IU/mL. The oocyte maturation process was carried out for 24 hours *in vitro*. This treatment was repeated in four replicates.

2. Stage 2. Addition of hypotaurine to maturation media

The effect of hypotaurine on oocyte maturation *in vitro* was examined. Oocytes were matured in M 199 media with four treatments, namely control, the addition of hypotaurine 2 mM, 4 mM, and 6 mM. The oocyte maturation process was carried out for 24 hours *in vitro*. This treatment was repeated in four replicates.

3. Stage 3. Addition of catalase and hypotaurine to maturation and fertilization media

Oocyte maturation and fertilization were carried out for 24 hours *in vitro*, in media supplemented with catalase and hypotaurine with the best treatment, control, catalase and hypotaurine concentrations and their combinations.

Oocyte collection

The ovaries used for this study were Bali cow ovaries collected at the abattoir, then placed in 0.9% NaCl solution to which 100 mg/mL streptomycin sulfate (Sigma-Aldrich, USA) and 100 UI/mL penicillin (Sigma-Aldrich, USA) were added. Oocyte collection was done using the slicing method. Oocytes surrounded by compact cumulus cells and having homogeneous cytoplasm were used in this study.

In Vitro maturation

The maturation medium included M 199, 0.3% bovine serum albumin, 10IU/mL follicle-stimulating hormone, 10IU/mL human chorionic gonadotrophin, and 50 µg/mL gentamicin (Sigma-Alderich, USA). IVM medium developed compact cumulus-surrounded oocytes with homogenous cytoplasm. Oocyte maturation occurred on petri dishes with 80µL drops (10-15 oocytes) coated with mineral oil (Sigma, Chemical Co. St. Louis MO, USA) in an incubator at 38.5°C and 5% CO_2 pressure for 24 hours (Hasbi et al. 2017).

In Vitro fertilization

After thawing at 37°C for 30 s, frozen semen was centrifuged at 1,800rpm for 5 min. The 1.5 x 10^6 spermatozoa/mL precipitate was added to fertilization medium after discarding the supernatant (Hasbi et al. 2020). Fertilization medium was placed in a petri dish as an 80µL drop coated with mineral oil (Sigma, Chemical Co. St. Louis MO, USA) and incubated for 30 minutes at 38.5°C and 5% CO_2 pressure. Oocytes developed for 24 hours were placed in IVF medium (Suzuki et al. 2000) and incubated at 38.5°C and 5% CO_2 pressure.

Nuclear maturation evaluation

The cumulus cells of developing oocytes were removed using 0.25% hyaluronidase (Sigma, USA). After denudation, oocytes were preserved in 1:3 acetic acid-ethanol absolute for 3 days. After 2% aceto-orcein staining, 25% acetic acid was added. Under a microscope (Zeiss Axio Image A2 with Zeiss Axiocam HRc camera, Germany), maturation was characterized by stage as GV, GVBD, MI and MII.

Fertilization rate evaluation

The fertilized oocytes cumulus cells were removed using 0.25% hyaluronidase (Sigma, USA). They were preserved for 3 days in a 1:3 solution of acetic acid and ethanol absolute after denudation. After 3 days, the preparations were stained with 2% acoto-orcien, washed off with 25% acetic acid, and observed with a microscope (Zeiss Axio Image A2 with Zeiss Axiocam HRc camera, Germany) to classify fertilization as two pronuclei (2 PN) or polyspermy (>2 PN).

Evaluation of hydrogen peroxide concentration

The concentration of H_2O_2 in oocytes was measured after 24 hours of IVM and IVF, using dichlorodihydroflurescein diacetate (DCHFDA) (sigma). DCHFDA stock solution was diluted in PBS to a concentration of 10µM. Oocytes were washed twice, then placed into PBS containing 10µM DCHFDA, then incubated at 38.5°C and 5% CO_2 pressure for 15 minutes. Next, new media was applied to a glass slide and coated with a cover glass. After 480 and 510nm excitation, a digital camera coupled to a fluorescence microscope captured fluorescence emission. Adobe Photoshop CS3 (Adobe Systems, San Jose, CA) transformed fluorescent photos to TIFF files, which were analyzed using imageJ 1.47 lumak. After color inversion, fluorescent pictures were counted by pixels. Fluorescence intensity indicates intracellular H_2O_2 levels (Gustina et al. 2019).

Statistical Analysis

Nuclear maturation, fertilization, and H_2O_2 concentration are mean±SEM. We used ANOVA to evaluate data. SPSS 27 was used for statistical analysis. The statistical significance criterion was $P<0.05$.

RESULTS

Addition of catalase to maturation media

Oocyte maturation is meiotic division from germinal vesicles (GV) resistant to metaphase-II (MII). Table 1 shows catalase added to maturation medium. This research found that 150IU/mL antioxidant catalase in maturation medium increased the proportion of oocytes reaching MII stage by 75.4%.

Table 1 reveals that antioxidant catalase does not promote nuclear maturation (P>0.05). A higher proportion of oocytes attain MII when maturation media include catalase. The number of MII oocytes increases with catalase concentration.

After color inversion, the pixels are counted to estimate H_2O_2 concentration. Fig. 1 shows DCHFDA-stained grapefruit oocyte fluorescent photomicrographs. Oocyte intracellular H_2O_2 concentration is seen in Fig. 2. H_2O_2 levels in oocytes treated with 100 and 150IU/mL catalase were considerably lower (P<0.05) than the control and 50IU/mL groups.

Addition of hypotaurine to maturation media

Hypotaurine is one of the antioxidants found in the fallopian tube and follicular fluid that reduces oxidative stress. The percentage of meiotic maturation of oocytes in maturation media supplemented with hypotaurine antioxidant in this study can be seen in Table 2.

Table 2 indicates that adding 4mM and 6mM hypotaurine to the maturation medium resulted in higher (P<0.05) rates (80.9 and 86.9%, respectively) compared to the control, but not substantially different from the 2mM oocytes reaching MII increased with hypotaurine content in maturation medium.

The addition of 4 and 6mM hypotaurine substantially reduced H_2O_2 levels (P<0.05) compared to the control group, but did not vary from the 2mM therapy. Fig. 3 shows DCHFDA-stained bovine oocyte fluorescent photomicrographs. Fig. 4 shows oocyte intracellular H_2O_2.

Addition of catalase and hypotaurine to maturation and fertilization media

Normal fertilization produces two pronuclei, while polyspermic oocytes contain more than two. Successful *in vitro* fertilization is measured by the creation of two or more. Table 3 shows the proportion of fertilized oocytes with catalase, hypotaurine and maturation and fertilization medium.

Table 3 indicates that oocytes fertilized on media supplemented with 6mM and 150IU/mL + 6mM have a significantly higher percentage of fertilized oocytes (P <0.05) compared to the control, but not significantly different from the 150IU/mL treatment. Mature and fertilized oocytes treated with catalase and hypotaurine *in vitro* had a greater proportion (78.6%).

Oocytes treated with 150IU/mL catalase (34.7%), 6mM hypotaurine (37.1%), or both showed substantially reduced H_2O_2 concentrations (P<0.05) compared to the control group (47.1%). Fig. 5 shows DCHFDA-stained grapefruit oocyte fluorescent photomicrographs. Fig. 6 shows oocyte intracellular H_2O_2.

DISCUSSION

Addition of catalase to maturation media

During *in vitro* culture, various factors can affect oocyte development, especially environmental conditions. High oxygen tension can induce ROS formation and inhibit oocyte growth rate (Lane and Gardner 2005). Manipulation of environmental conditions *in vitro* culture leads to downregulation of cells caused by increased levels of ROS (Yu et al. 2014; Agarwal et al. 2022). Goto et al. (1993) stated that ROS production is higher in *in vitro* conditions than *in vivo.* Under normal conditions, the balance of ROS

Table 1: Maturation rate of Bali cow oocytes with the addition of antioxidant catalase

Treatments	Oocytes	GV (%±SEM)	GVBD (%±SEM)	MI (%±SEM)	MII(%±SEM)
Control	105	4(3.6±1.5)	11(9.5±2.6)	19(18.0±2.0)	71(68.8±5.5)
50IU/mL	102	3(2.5±1.3)	8(7.1±3.0)	19(18.1±1.7)	72(72.2±5.6)
100IU/mL	106	2(1.7±11.0)	10(8.3±3.1)	17(15.9±1.7)	77(74.0±4.8)
150IU/mL	101	2(1.6±1.1)	8(7.3±2.5)	15(15.6±2.8)	76(75.4±3.3)

Description: Germinal vesicle (GV), Germinal vesicle breakdown (GVBD), Metaphase I (MI), Metaphase II (MII)

Table 2: Maturation rate of Bali cow oocytes with the addition of hypotaurine antioxidant

Treatments	Oocyte Count	Core Maturation Level			
		GV(%±SEM)	GVBD(%±SEM)	MI (%±SEM)	MII(%±SEM)
Control	103	2(2.0±1.3)	10(10.3±2.3)^{a}	24(23.3±1.8)^{a}	66 (64.3±3.0)^{a}
2mM	106	1(1.1±1.1)	7(7.8±2.6)^{ab}	21(18.4±2.2)^{ab}	79(72.6±3.4)^{ab}
4mM	101	0(0.0±0.0)	2(2.4±1.6)^{b}	16(16.6±2.0)^{b}	83(80.9±3.3)^{b}
6mM	110	0(0.0±0.0)	1(0.8±0.8)^{b}	13(12.2±1.37)^{b}	96(86.9±1.8)^{b}

Description: ^{a,b} different superscripts in the columns indicate significant differences (P<0.05). *Germinal vesicle breakdown* (GVBD), *Metaphase I* (MI), *Metaphase II* (MII)

Table 3: Fertilization rate of oocytes with addition of antioxidants catalase, hypotaurine and combination of catalase and hypotaurine

Treatments	Oocyte Count	Fertilization Rate		
		2 PN (%±SEM)	3 PN (%±SEM)	Total(%±SEM)
Control	107	72(67.2±1.3)^{a}	0(0.0±0.0)	72(67.2±1.3)^{a}
150IU/mL (catalase)	102	73(71.9±1.4)^{ab}	1(0.8±0.8)	74(72.7±1.4)^{ab}
6 mM (hypotaurine)	104	76(72.9±1.1)^{b}	2(2.0±1.2)	78(75.0±1.7)^{b}
150IU/mL+6 mM (combination)	109	84(76.8±2.2)^{b}	2(1.7±1.1)	86(78.6±2.7)^{b}

Description: ^{a,b} Different superscripts in the columns indicate significant differences (P<0.05). *2 pronuclei* (2 PN), *polyspermi* (3PN)

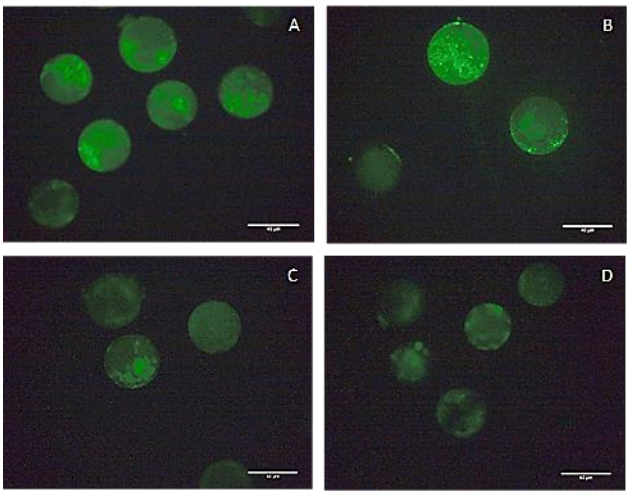

Fig. 1: Fluorescent photomicrographs of bovine oocytes stained with 2',7'- dichlorodihydrofluorescein, supplemented with catalase. Control (A), 50IU/mL (B), 100IU/mL (C), 150IU/mL (D)

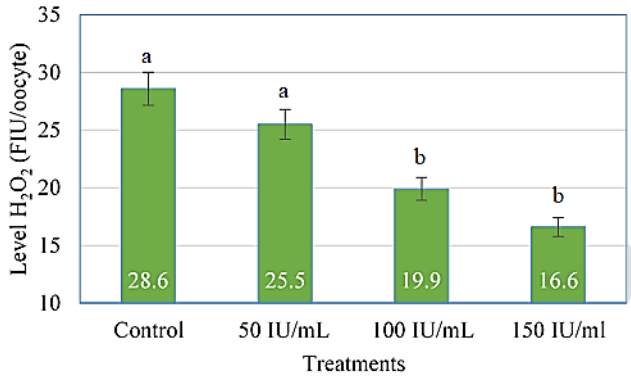

Fig. 2: Hydrogen peroxide concentration of catalase supplemented bovine oocytes; FUI: value represents the unit of fluorescence intensity. [a,b] Different superscripts indicate significant differences (P<0.05)

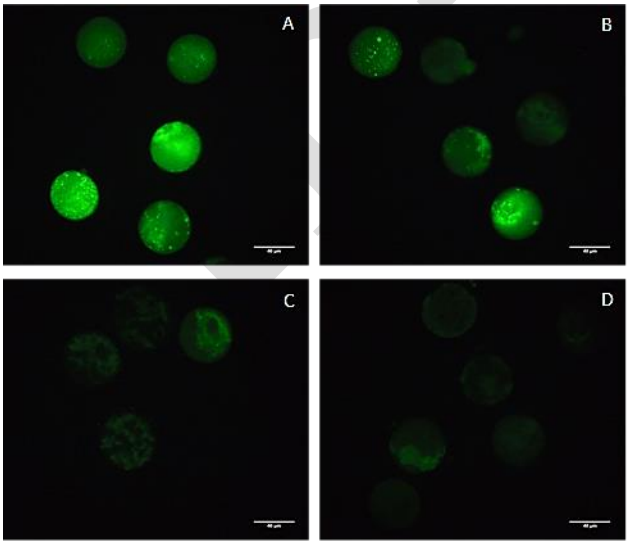

Fig. 3: Fluorescent photomicrographs of bovine oocytes stained with 2',7'- dichlorodihydrofluorescein, supplemented with hypotaurine. Control (A), 2mM (B), 4mM (C), 6mM (D)

can be maintained by enzymatic and non-enzymatic antioxidants. However, during *in vitro* culture, antioxidant production naturally decreases, resulting in increased ROS production (Takahashi 2012; Zhang et al. 2006). Thus, several studies show that adding antioxidants to the culture medium improves oocyte maturation and fertilization *in vitro*.

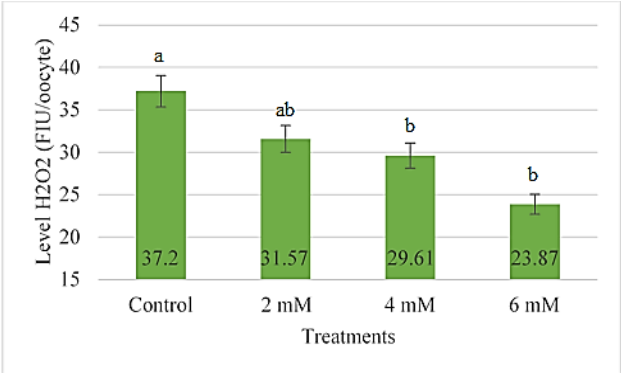

Fig. 4: Hydrogen peroxide concentration of hypotaurine-supplemented bovine oocytes; FUI: value represents the unit of fluorescence intensity. [a,b] Different superscripts indicate significant differences (P<0.05).

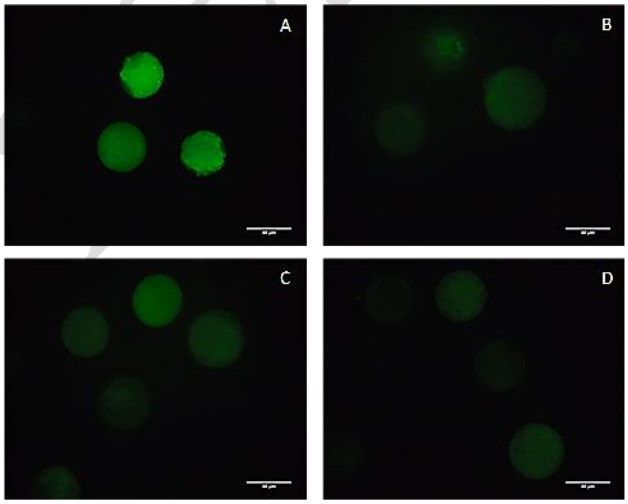

Fig. 5: Images of bovine oocytes stained with 2',7'-dichlorodihydrofluorescein, catalase, hypotaurine, and combination. Control (A), 150IU/mL (B), 6mM (C), 150IU/mL 6mM (D)

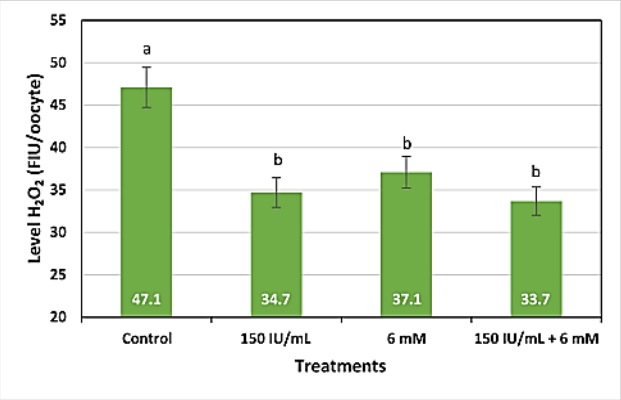

Fig. 6: Hydrogen peroxide concentration of catalase and hypotaurine-supplemented bovine oocytes. FUI: value represents the unit of fluorescence intensity. [a,b] Different superscripts indicate significant differences (P<0.05).

This research found that catalase did not raise MII oocytes (P>0.05) compared to the control. The membrane may prevent catalase from crossing (Ali et al. 2003). According to Nasr-Esfahani et al. (1990), catalase-supplemented *in vitro* culture conditions did not impact endogenous H_2O_2 generation. So extracellular catalase is more active. This research found that catalase in the maturation medium reduced intracellular H_2O_2 concentrations. The extracellular action of catalase may reduce intracellular H_2O_2. Catalase reduces oxidative stress by converting H_2O_2 to water and oxygen (Von Mengden et al. 2020). Lipid peroxidation chain reaction may diminish, lowering H_2O_2 and other ROS damage (Guerin 2001). Thus, the addition of catalase is a strategy to prevent intracellular ROS formation (Circu and Aw 2010; Damayanti et al. 2022). Catalase-supplemented maturation medium may protect oocytes from oxidative damage.

Addition of hypotaurine to maturation media

The addition of 4 and 6mM hypotaurine to the maturation media in this study can increase the percentage of oocytes reaching the MII stage significantly (P>0.05) against the control. This is consistent with the ability of hypotaurine to neutralize hydroxyl radicals (Guerin 2001; Partyka et al. 2017). Hypotaurine also functions as an amino acid that plays an important role in reducing DNA fragmentation triggered by oxidative stress (Fellman et al. 1987). Through a non-enzymatic reaction mechanism during interaction with ROS (Conrado et al. 2021), the presence of hypotaurine in the maturation medium can reduce the intracellular H_2O_2 content. The decrease in the concentration of H_2O_2 added by hypotaurin in the maturation media is due to the function of hypotaurin as an aminosulfinic acid that plays a role in reducing the production of ROS. Hypotaurine can react with H_2O_2 to form taurine (Grove and Karpowicz 2017) which acts as an antioxidant by removing hydroxyl radicals (Chen et al. 2020). The increase in the level of hypotaurine added to the maturation medium is in line with the decrease in intracellular H_2O_2 concentration in oocytes. This shows that hypotaurine can reduce H_2O_2 thus increasing cell viability, by increasing oocytes reaching the MII stage.

Addition of catalase and hypotaurine to maturation and fertilization media

The fertilization rate was assessed based on the number of oocytes that had 2 or more pronuclei after fertilization. The addition of 6mM hypotaurine and the combination (150IU/mL catalase + 6mM hypotaurine) were able to increase the fertilized oocytes compared to the control. However, the addition of catalase 150IU/mL could not increase the fertilized oocytes. Based on the results of this study, the addition of hypotaurine and the combination of catalase and hypotaurine have a positive effect compared to catalase. The maturation stage, oocytes will undergo nuclear maturation and cytoplasmic maturation (Van Den Hurk and Zhao 2005). Cytoplasmic maturation in oocytes is needed to prevent polyspermia, decondensation of spermatozoa and help pronucleus formation. At the fertilization stage, sperma quality also plays an important role in the success of *in vitro* embryo production (Rahmatullah et al. 2022). Good quality is characterized by high viability, normal morphology, and progressive mortality (Dcunha et al. 2022). Process capacitation and acrosome reaction in sperm affect the formation of male pronucleus in the oocyte cytoplasm (Iskandar et al. 2019). This shows that oocyte maturation, capacitation and acrosome reaction affect the success of oocyte fertilization. The addition of catalase to the maturation and fertilization media plays a role in reducing H_2O_2. Catalase also plays a role in improving motility, plasma membrane integrity, mitochondrial potential and low ROS (Arslan et al. 2019). In addition, the addition of hypotaurine to the maturation and fertilization media serves to reduce oxidative stress (Chen et al. 2020). Hypotaurine can protect the phospholipid layer, reduce intracellular free radical levels and increase antioxidant enzymes (Zhang et al. 2021). Hypotaurine also plays a role in increasing capacitation, motility and fertilization ability (Bucak et al. 2013). These functions can reduce the percentage of chromatin decondensation, DNA fragmentation and sperm nucleus vacuolization (Pons-rejraji et al. 2021). Therefore, the addition of catalase, hypotaurine and a combination of both in the maturation and fertilization media can increase the fertilization rate of oocytes *in vitro*. In addition, the addition of catalase, hypotaurin and the combination of catalase and hypotaurin can reduce the intracellular H_2O_2 content. The decrease in H_2O_2 concentration is due to the role of catalase catalyzing H_2O_2 into H_2O and O_2 (Von Mengden et al. 2020). Hypotaurin can react with superoxide anions to form new prositaurine molecules (Conrado et al. 2021). In addition, hypotaurine can also react with H_2O_2 to form taurine (Grove and Karpowicz 2017). The results of this study, the combination of catalase and hypotaurine showed the lowest percentage compared to other groups. This shows that the combination of catalase and hypotaurine can maintain the condition of the culture environment to be neutral so that it can increase oocyte fertilization.

Conclusion

The research found that antioxidant catalase at 150IU/mL reduces H_2O_2 but does not raise MII oocytes. The addition of 6mM hypotaurine in the maturation media can increase the percentage of oocytes reaching the MII stage followed by a decrease in H_2O_2. The combination of catalase and hypotaurine in maturation and fertilization media can increase the percentage of fertilized Bali cattle oocytes and reduce H_2O_2.

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