Effect of the Addition of Insulin-Transferrin-Selenium on In Vitro Maturation and Fertilization of Bali Cattle Oocytes

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ABSTRAK

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Penelitian ini dilakukan untuk mengetahui pengaruh penambahan Insulin Transferrin Selenium (ITS) pada medium terhadap tingkat maturasi dan fertilisasi oosit sapi Bali secara *in vitro*. Ovarium sapi Bali disayat untuk menghasilkan oosit, lalu oosit dikoleksi dan diseleksi berdasarkan kualitasnya. Oosit tersebut lalu dimaturasi 24 jam dan difertilisasi 18 jam di dalam inkubator 5% CO2 dan 38,5°C. Oosit diwarnai dengan aceto orcein 2%, lalu diamati di bawah mikroskop. Penelitian ini menggunakan Rancangan Acak Lengkap (RAL) dengan empat perlakuan penambahan ITS (P0 kontrol; P1 (5 ng/ml); P2 (10 ng/ml); dan P3 (15 ng/ml)) dan 4 ulangan. Parameter yang diamati yaitu tahap tingkat maturasi oosit yang terdiri dari germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase-I (M-I) dan metaphase-II (M-II), tingkat fertilisasi (0 pronukleus (0 PN), 1 pronukleus (1 PN), 2 pronukleus (2 PN) dan lebih dari 2 pronukleus (>2 PN)). Hasil penelitian menunjukkan bahwa persentase tingkat maturasi oosit tertinggi cenderung pada tahap M-II yaitu dicapai oleh oosit P1 dengan pemberian ITS sebanyak 5 ng/ml sedangkan persentase tingkat fertilisasi tertinggi pada tahap PN-2, yaitu dihasilkan oleh oosit P3 dengan pemberian ITS sebanyak 15 ng/ml. Kesimpulan dari penelitian ini adalah bahwa pemberian ITS sebanyak 5 ng/ml cenderung menghasilkan tingkat maturasi yang terbaik dan untuk tingkat fertilisasi yang terbaik cenderung pada pemberian ITS sebanyak 15 ng/ml.

Kata Kunci: Ovarium Sapi Bali, Fertilisasi, Insulin-Transferrin-Selenium, Maturasi

ABSTRACT

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This study was conducted to determine effect of the addition of Insulin Transferrin Selenium (ITS) on in vitro maturation and fertilization of Bali cattle oocytes. Bali cattle ovary is sliced to obtain oocytes, then oocytes were collected and selected based on their quality. Oocyte then maturated for 24 hours and fertilized for 18 hours in an incubator of 5% CO2 and 38.5°C. Oocyte was stained with 2% acetoorcein, then observed under a microscope. This study was done based on a Completely Randomized Design (CRD) with four treatments of ITS addition (P0 control; P1 (5 ng/ml); P2 (10 ng/ml); and P3 (15 ng/ml)) in 4 replications. Parameters observed were the stage of oocyte maturation level consisting of germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase-I (MI) and metaphase-II (M-II), fertilization rate (0 pronucleus (0 PN) , 1 pronucleus (1 PN), 2 pronucleus (2 PN) and more than 2 pronucleus (>2 PN)). Results showed that the highest percentage of oocyte maturation rate tends to be in the M-II stage, which is achieved by P1 oocytes with ITS addition as much as 5 ng/ml while the highest percentage of fertilization rate at PN-2 stage, which was produced by P3 oocytes with ITS addition as much as 15 ng/ml. It is concluded that the addition of ITS at 5 ng / ml tends to produce the best maturation rate and for the best level of fertilization tends to be as much as 15 ng / ml of ITS addition.

Key words: Bali Cattle Ovary, Fertlization, Insulin-Transferrin-Selenium, Maturation,

INTRODUCTION

At the time of in vitro maturation, oocytes develop metabolism with the condition of O_2 at higher concentrations. This condition triggers an increase in free radical production (Reactive Oxygen Species/ROS) resulting in oxidative stress conditions. This high level of ROS can damage cell membranes due to membrane lipid perioxidation. An enzymatic antioxidant system found in mammalian cells, namely superoxside dismutase, glutathione peroxidase, and catalase can function as ROS scavenger (Cetica et al. 2001). Therefore, the addition of antioxidants is needed in the process of oocytes maturation to inhibit cell damage due to ROS and increase the rate of cell growth in oocytes.

Antioxidant supplements such as α-tocopherol, glutathione (GSH) and Insulin Transferrin Selenium

(ITS) were added to the oocytes maturation medium with the aim of scavenging free radicals. Research (Kim et al. 2005; Lee et al. 2005) showed administration of insulin and insulin like growth factor (IGF) into the medium, could increase oocytes growth potential, the level of embryo cleavage during in vitro maturation (IVM), and in vitro Cultured (IVC) in pigs.

Insulin Transferrin Selenium (ITS) act as antioxidants in biological systems in rats (Wu et al. 2007; Gutteridge 1986). The use of ITS as a supplement has been carried out for rat oocytes (De La Fuente et al. 1999), ITS and epidermal growth factor (EGF) succeeded in managing prepubertal rat oocytes and preantral follicles by 92.2% (Gao et al. 2007), goats (Herrick et al. 2004), pig oocytes (Jeong et al. 2008) significantly increase the concentration of glutathione. The combination of ITS can be used both in media maturation and complex and non-complex fertilization, ITS is a supplement that can stimulate oocytes growth. However, studies and scientific information regarding the effect of Insulin Transferrin Selenium (ITS) in Bali cattle are still very limited.

Problems arising from the low quality of the results of in vitro maturation IVM and in vitro fertilization (IVF) oocytes by cell damage due to oxidative stress caused by increased production of free radicals or Reactive Oxygen Species (ROS) in vitro. Free radicals are atoms or molecules that have electrons that are not paired with orbitals (Gutteridge 1986). In order to get stability chemically, free radicals cannot maintain their original form for long periods and soon bind to the surrounding material. Free radicals will attack stable molecules the closest one and takes electrons, the substances that electrons take up will become radical also free so that it will start a chain reaction, which eventually couse cell damage (De La Fuente et al. 1999). Free radical activity can be reduced by administering antioxidants (Gao et al. 2007). Antioxidants are compounds that can inhibit, delay, prevent or slow down oxidation reactions even in small concentrations. Bali cattle is a germ plasm asset of Indonesian native cattle with some specific advantages, among them: very good reproductive properties and high fertility so that the addition of ITS in the media of maturation and fertilization is expected to provide a better response. Also the addition of antioxidant supplementation materials ITS into the maturation and fertilization medium aims to prevent the effects of ROS, so that the oocytes metabolic process can take place normally.

ITS is a media supplement in in vitro maturation that suppress the influence of free radical compounds that trigger the oxidative environment. So this study was conducted to evaluate the effect of ITS on the maturation and fertilization of Bali cattle oocytes in vitro.

MATERIALS AND METHODS

Sample collection

Bali cattle ovary obtained from Tamangapa Animal Slaughter house, Makassar City, South Sulawesi province and taken to the Fertilization and Embryo In Vitro Laboratory of Hasanuddin University with a distance of 10 km in a solution of 0.9% NaCl plus 100 IU/mL of penicillin and 100 µg/mL of streptomycin sulfate. The number of ovaries is ten out of five Bali cattle. Incubator temperature used is 37° C, 5% CO_2.

Oocytes collection

Ovaries obtained from Makassar Tamangapa RPH use physiological solutions of 0.9% NaCl first rinsed twice on 0.9% NaCl. Washed and dried, glass dishes were prepared, then filled with media of phosphate buffered saline (PBS). The ovaries were placed on the glass dish, then chopped. Oocytes are collected in a dish that has been filled with collection media (Phosphate Buffered Saline/PBS + 10% Fetal Bovine Serum/FBS). Only oocytes of A and B quality were used. Quality A oocyte (having a uniform and compact cumulus with surrounded by five or more layers of cumulus cells), quality B oocytes (characterized by uniform oocytes and having cytoplasm the dark with a complete complement of corona radiata but surrounded by no more than five layers of cumulus cells).

In Vitro Oocytes Maturation

Collected oocytes were washed in each maturation medium twice, then ripening in TCM-199 (Sigma, USA) supplemented with 0.3% bovine serum albumin (BSA), 10 IU/ml pregnant mare serum gonodrptropin (PMSG) (Intergonan, Intervet Deutschland GmbH), 10 IU/ml human chorionic gonadrotropin (hCG) (Chorulon, Intervet international BV Boxer-Holland), 50 µg/ml gentamycin (Sigma, USA), and the addition of Transferrin Selenium Insulin (ITS) with different concentrations (controls (0 ng/ml), 5 ng/ml, 10 ng/ml and 15 ng/ml) (Romar & Funahashi 2006; Shirazi & Sadeghi 2007).

In Vitro Fertilization

Stage of fertilization based on (Suzuki et al. 2000) is as follow:

Frozen semen straw was thawed at 37°C for 20 seconds, then put it into the prepared spermatozoa washing medium to be centrifuged for 5 minutes at 1800 rpm of speed. The supernatant was discarded and then the deposited sperm was added with the second washing medium. It was centrifuged again with the

same time and speed. The centrifuged supernatant was removed and the sperm was diluted with fertilization media and made in the form of a drop in the fertilization dish. The coating used ±3 ml mineral oil to cover the entire surface of the fertilization media. Mature oocytes were removed from the incubator, then taken and placed on the media wash and washed twice. Washed oocytes were placed in a fertilization dish containing of sperm drop which then incubated for ≥16 hours.

Fixation

Fixation process was started by removing fertilization dish from incubator after ≥16 hours of fertilization. The fertilized oocytes were then removed and washed three times (all sperms were not involved and only a few cumulus cells surround the oocytes). The fertilized oocytes are washed again 2-3 times and then moved to become preparations and glue the objects glass and glass cover using vaselin (adhesive). The fixation was used ethanol: acetic acid for three days. Then the preparation was rinsed using absolute ethanol for one hour.

Cell Coloring

Cell staining preparations used 2% acetone orcein then rinse again with 25% acetic acid. Oocyteswere examined under an inverted microscope to observe the maturation and fertilization rates.

Observed parameters

The parameters observed in this study were the level of oocyte maturation and the rate of fertilization. Oocyte maturation rate according to (Sonjaya et al. 2016) was germinal vesicle (GV) phase which was characterized by the presence of a nuclear membrane and a nucleoli clearly visible on the edge; Germinal Vesicle Breaking Down (GVBD) phase which was characterized by the tearing of the core membrane so that the nucleoli not clearly visible. Metaphase-I (M-I) phase was characterized by the presence of homologous chromosomes that were paired and lined up in the equatorial plane. Metaphase-II (M-II) phase was characterized by the presence of a polar body I and the same chromosome arrangement as the M-I stage, anaphase and telophase phases.

Oocyte maturation rates may be calculated according to the formula below:

$$\frac{The\ number\ of oocytes\ under\ going\ as\ tage\ of\ maturation}{The\ number\ of\ oocytes\ being\ saturated} \text{X}100\%$$

Meanwhile, fertilization rates according to Syaiful et al. (2011) was fragmented oocytes or oocytes that do not achieve metaphase II development (0 PN); oocytes that have one pronucleus (1 PN) consisting only of female pronucleus; oocytes that have two pronucleus (2 PN) consisting of male and female pronucleus; fertilized oocytes that have two or more pronucleus (> 2 PN).In vitro fertilization rates may be calculated according to the formula below:

$$\frac{The\ number\ of oocytes\ under\ going\ as\ tage\ of fertilization}{Number of fertilized\ oocytes} \text{X}\ 100\%$$

Data analysis

The level of maturity and oocyte fertilization were analyzed by Completely Randomized Design (CRD) Contingency Table with the following formula (Steel & Torrie 1993):

$$\text{X}^2 = \frac{[(BxC) - (AxD)]^2\ \text{E}}{(A + C)(B + D)(A + B)(C + D)}$$

TREATMENT	X	Y	
ITS 1	A	B	A+B
ITS 2	C	D	C+D
	A+C	B+D	A+B+C+D=E

Table 1. Bali cow oocytes maturation level with Insulin Transferrin Selenium (ng/ml) addition in different media

Treatments (ITS addition)	Oocytes count	Maturation level (%)			
		GV	GVBD	M-I	M-II
Control (0 ng/ml)	36	0.00 ± 0.00[a]	9.00 ± 10.39[ab]	25.75 ± 6.13	26.00 ± 0.00[b]
5 ng/ml	26	0.00 ± 0.00[a]	0.00 ± 0.00[a]	17.50 ± 12.26	25.25 ± 9.91[b]
10 ng/ml	34	18.00 ± 0.00[b]	11.00 ± 13.11[ab]	19.25 ± 14.22	18.00 ± 0.00[ab]
15 ng/ml	32	9.00 ± 10.39[c]	20.00 ± 4.00[b]	22.00 ± 4.61	13.50 ± 9.00[a]

GV= germinal vesicle; GVBD= germinal vesicle break down; M-1=metaphase-I (MI); M-II= metaphase-II; [a,b]different superscript in the same column show real differences (P≤0.05)

RESULTS AND DISCUSSION

Level of Bali cow oocytes maturation with addition of Insulin Transferrin Selenium (ITS) in maturation media

The observation of the maturation rate with the addition of different level of Insulin Transferrin Selenium (ITS) in maturation media is presented in Table 1. The analysis results showed that the addition of ITS to oocytes had no significant effect (P> 0.05) on oocytes maturation rates. However, the addition of ITS 0 ng/ml-5 ng/ml into maturation media in this study tends to have a higher maturation rate compared to the addition of ITS 10 ng/ml and 15 ng/ml. This means that the addition of ITS of 0 ng/ml-5 ng/ml into the medium of maturation is sufficient to increase the oocytes maturation rate and support the development of oocytes to reach the stage of MII.

The number of oocytes used for each treatment was 40 oocytes, but at the time of observation there were several oocytes which were lost where in P0 the remaining oocytes were 36, P1 (26 oocytes), P2 (34 oocytes) and P3 (32 oocytes). By the glass cover at the time of fixation it is not well pressed so that when the cell staining occurs, some oocytes lost. Also the treatment of an increased ITS dose causes an irregular decrease in oocytes. This is possible, ITS content can increase GSH concentration.

The tendency of different oocytes maturation percentage shows that ITS supplementation in maturation media has an influence on oocytes maturation and can be used as an indicator of increasing cell life force, with increasing oocytes to stage M-II. This means that supplementation of ITS 5 ng/ml into the medium of maturation can reduce the rate of apoptosis. Whereas with ITS supplementation of 10 ng/ml-15 ng/ml, oocytes up to M-II stage tend to decrease, this is because the dose given exceeds the good dose so that free radicals at maturation that should be suppressed actually have an impact on oocyte reduction until stage M-II.

ITS supplementation on the maturation medium can reduce the oxidation reaction of ROS, because the content of selenium in the ITS medium is the main element of the antioxidant gluthathione peroxidase (GSx) and reduced gluthathione (GSH) which serves to prevent damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides (Djuwita et al. 2012). According to Hwang et al. (1992) Glutathione (GSH) is considered the most abundant molecule among endogenous antioxidants. GSH is a reduced peptide consisting of three-residues (γL-glutamyl-L-cysteinyl glycine) which can donate an electron with the consequence that two electron donating GSH molecules form oxidized GSSG. In humans, GSH is almost uniquely present in a quite high concentration (1–10 mM) which allows to scavenge.

ROS either directly or indirectly Werdhany (1999), GSH is an antioxidant that play a role in preventing the formation of new free radicals and reducing existing free radicals. In addition, the transferrin and selenium contents are important for the GSH peroxidase catalyst activity (Cerri et al. 2009).

Cytoplasm of oocytes strongly supports the spread of organelles and interactions between other organelles, as well as the presence of compact cumulus cells that can support the maturation of oocytes through metabolites produced and secreted through a gap junction mechanism to oocytes cells (De Loos et al. 1989).

In this study only oocytes that had complex cumulus (categories A and B) were used in the in vitro maturation process (Figure 2). According to (De Loos et al. 1989) the oocytes that well included were compact, multilayered and compact cumulus cells, homogeneous ooplasm, total bright and transparent COC (Cumulus Oocytes Complex). The presence of cumulus cells supports the occurrence of oocytes maturation in vitro to stage M-II and related to cytoplasmic maturation (Lapathihis et al. 2002). In providing nutrients for oocytes and helping synthesize proteins to form a pellucid zone at prophase stage. Egg cells without cumulus after being matured, might lost many proteins while in egg cells with intact cumulus, protein will survive.

During in vitro maturation of bovine oocytes the presence of cumulus cells surrounding the oocytes is very helpful to the development of blastocysts (Boediono & Suzuki 1996).

Observations on Bali cattle oocytes ranging from un-denaturated oocytes to oocytes after staining with various levels of core maturation can be seen in Figure 1 and Figure 2.

Factors that support the success of oocytes core maturation (Zheng & Sirard 1992) are the expansion of the cumulus maturation cells of the nucleus that reaches M-II and cytoplasmic maturation. According to Motlík & Fulka (1976) the cumulus cells that surround the oocytes will be wide and brightly colored. Oocytes have reached maximum maturation and are ready to fertilize if they have reached stage M-II in the process of meiotic division (Figure 2).

The meiosis process begins with the GV stage, which is characterized by a clear core membrane and a clear colored necloulus ring followed by the rupture of the core stage or GVBD, necloulus disappears and the one pole body has been formed (Tsafriri 1985), while stoplasma maturation includes the addition of yellow grains eggs in the cytoplasm, formation of the pellucid zone sheath, and the formation of cortical granules (Djuwita et al. 2000).

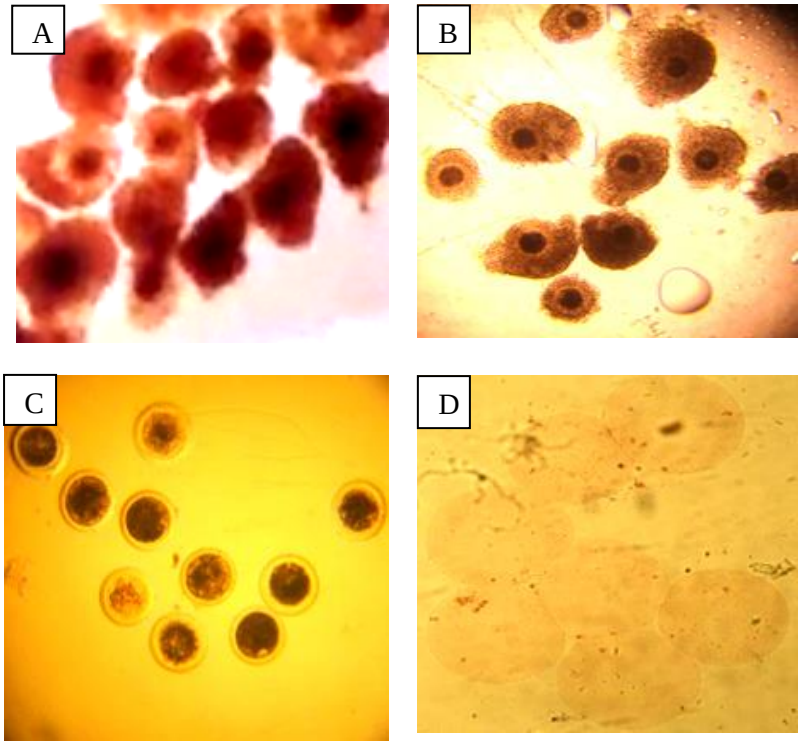

Figure 1. Bali cow oocytes changes from un-denaturated to after staining with various levels of core maturation. (A) oocytes before maturation, (B) oocytes after maturation,(C) oocytes after denudation, (D) oocytes after staining

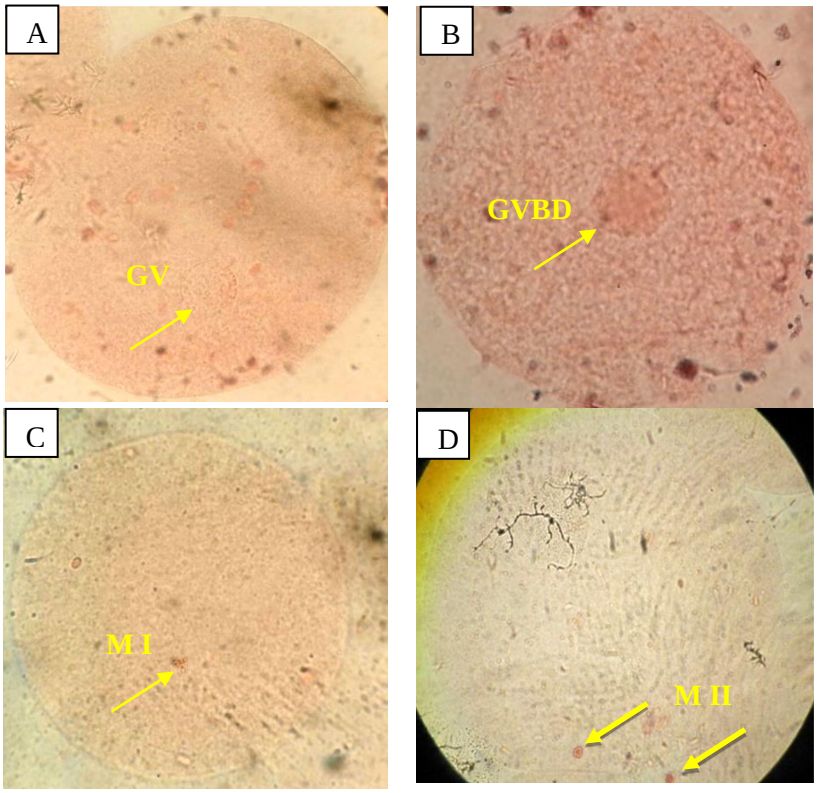

Figure 2. Bali cow oocytes maturation core status after in vitro maturation. Arrow marks indicate core status at stage (A) oocytes vesicle germinal stage, (B) oocytes vesicle germinal stage breaks down, (C) oocytes stage metaphase-I, (D) mature oocytes metaphase-II stage

Table 2. Bali cow oocytes pronucleous formation (%) and fertilization rates with different level of Insulin Transferrin Selenium addition

Treatment (ITS addition)	Oocytes count	Pronucleusformation(%)				Fertiluzation rate
		0 PN	1 PN	2 PN	>2 PN	
Control	36	22.75±16.35^{b}	9.00±10.39	11.00±13.11	17.50±12.26	26.00±0.00^{a}
5 ng/ml	26	0.00±0.00^{a}	9.00±10.39	22.00±4.61	17.50±12.26	31.00±6.27a^{b}
10 ng/ml	34	9.00±10.39$_{ab}$	9.00±10.39	23.75±7.22	22.00±4.61	34.25±7.88^{ab}
15 ng/ml	32	0.00±0.00^{a}	9.00±10.39	20.00±4.00	27.50±7.14	36.00±3.46^{b}

PN= pronucleous; ^{a,b}different superscripts in the same column show real differences (P≤0.05)

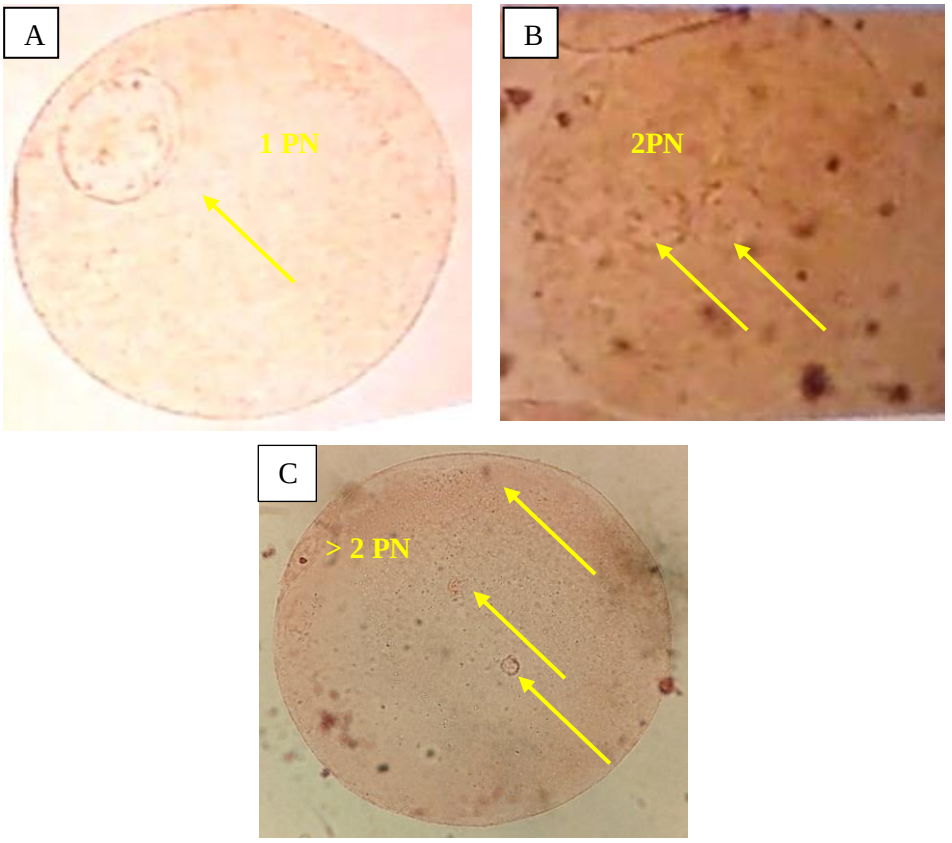

Figure 3. Bali cow pronucleous formation. PN= pronucleous; (A) oocytes stage 1 pronucleus; (B) fertile oocytes stage 2 pronucleus; (C) fertile oocytes stage >2 pronucleus

Bali cow oocytes fertilization rate with different addition of Insulin Transferrin Selenium (ITS)

The results of observing the degree of fertilization with the addition of different Insulin Transferrin Selenium (ITS) Insulin are presented in Table 2.Results of the analysis showed that the effect of the addition of ITS on the oocytes had no significant effect (P> 0.05) on the level of oocytes fertilization. However, with the addition of ITS 15 ng/ml the fertility rate tends to be higher than the addition of 5 ng/ml and 10 ng/ml. This means that the addition of ITS of 15 ng/ml increased the level of oocytes fertilization and was better than other treatments. Rusiyantono et al. (2000) obtained a value of 65.4% in tissue culture medium (TCM 199)+Essensial media, while Djuwita et al. (1995) obtained a value of 31.7% in TCM 199+ media for fetal serum (EFS) and 25.6% in TCM 199+FCS media.

Meanwhile, the role of ITS for P2 and P0 and those who were not given ITS (control) was higher but the fertility rate was low. This is thought to be related to the role of ITS which can optimize cell growth better when fertilized. Furthermore, it is also suspected that the low level of fertilization in the P2 medium supplemented with ITS or without the addition of ITS results in differences in protein metabolism needed to improve oocytes competency (Orsi & Leese 2004).

Fertilization failure is characterized by the presence of one pronucleus and in this study varied. Fertilization failure is influenced by several factors, among others: the level of oocytes maturation in both the nucleus and cytoplasm is incomplete (Boediono et al. 2000); adequate and failure of spermatozoa to experience condensation in the oocytes cytoplasm causing a failure of male pronucleus formation (Crozet et al. 1995). The incidence of polyspermi in this study varied. The incidence of polyspermi may be caused by various factors including the concentration of spermatozoa (Nadir et al. 1993; Long et al. 1994), the length of incubation of spermatozoa and oocytes (Long et al. 1994) and not perfect blockade of vitelin (Dandekar & Talbot 1992).

Another factor that also affects the ability of in vitro fertilization is the production of ROS. Dead spermatozoa produce ROS causing membrane lipid peroxidation, reducing membrane fluidity and sperm function. High ROS destroys the metabolism of spermatozoa in the media of in vitro fertilization. Kim et al. (2002) said ROS increased under in vitro conditions using 5% CO_2.

Fertilized oocytes are characterized by the formation of PN (Figure 3). Oocytes that have undergone a maturation process for 24 hours are then fertilized for 18 hours in an incubator with CO_2 and the same temperature when matured. Before the fertilization process, the sperm is frozen first in thawing and diluted on the fertilization medium.

CONCLUCIONS

Addition of Insulin Transferrin Selenium (ITS) to mediums of 10-15 ng/ml did not significantly influence the maturation and fertilization of Bali cattle oocytes in vitro.

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