NUSANTARA BIOSCIENCE
Vol. 9, No. 2, pp. 202-208
May 2017

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n090216

Fertilization and development of mice (*Mus musculus*) embryo in vitro after supplementing the extract of *Pandanus conoideus*

KARTINI ERIANI[1,♥], PUTRI LAILAN TIFANI[1], SYAHRUDDIN SAID[2]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala. Jl. Syeh Abdurrauf No. 3, Banda Aceh 23111, Aceh, Indonesia. Tel. +62-85277060751, ♥email: kartini_eriani@unsyiah.ac.id

[2]Research Center for Biotechnology, Indonesian Institute of Sciences, Jl.Raya Bogor Km 46 Cibinong, Bogor 16911, West Java, Indonesia

Manuscript received: 31 August 2016. Revision accepted: 20 April 2017.

Abstract. *Eriani K, Tifani PL, Said S. 2017. Fertilization and development of mice (*Mus musculus*) embryo* in vitro *after supplementing the extract of* Pandanus conoideus*. Nusantara Bioscience 9: 202-208.* An extract of red fruit (ERF/*Pandanus conoideus* Lam.) contains beta-carotene and alpha-tocopherol, i.e. antioxidant compounds group which can stop the formation of free radicals during in vitro fertilization. Reactive oxygen species (ROS) is a free radical which resulted in an oxygen derivate. This research aimed to determine the effect of ERF supplementation on fertilization rate and embryo in vitro development of mice. The study used Completely Randomized Design (CRD) with three group of treatments (ERF dosage supplement: 0 mL, 0.05 mL, and 0.1 mL) and three replications. Each dosage was added to five mice in 7 days. In total, 45 samples were used. Research procedure included extraction of *P. conoideus* Lam, treatment on the sample, superovulation, preparation of sperm, oocyte collection, *in vitro* fertilization, and in vitro culture. Parameters observed were the quality of oocyte, the fertilization rate of mice and the development stage of in vitro pre-implantation mice embryo. Acquired data analyzed quantitatively using One Way ANAVA and Duncan's Multiple Range Test (DMRT) at 5% significance level. The result showed that all treatments did not affect significantly ($p>0.05$) on the quality of oocyte, in vitro fertilization of oocyte and in vitro early development of embryo. Suggested for further research about the improvement technique of *in vitro* fertilization and early embryo development.

Keywords: Extract *Pandanus conoideus*, in vitro fertilization, early embryo development, in vitro culture

INTRODUCTION

The development of biotechnology nowadays plays a vital role in many areas such as agriculture, farming, fishery, medicine, and health. Gordon (1994) stated that one of the advantages of biotechnology in farming is increasing farming product including reproductive technology such as artificial insemination, embryo transfer, cryopreservation, in vitro fertilization (IVF), spermatozoa sexing, embryo cloning, genetic engineering, and other forms of biotechnology in veterinary area. One of the present reproductive technology that has been explored widely is in vitro embryo production (IVEP). In vitro embryo production is a form of assisted reproductive technology (ART) consisted of in vitro maturation (IVM), in vitro fertilization and in vitro culture (IVC). Rabbit, mice, human, pig, cow and sheep embryos have been successfully produced (Hafez and Hafez 2000).

The success of in vitro fertilization depends a lot on the quality of oocytes. Oocytes found in female reproductive duct will have good qualities if it is free of free radicals. Female reproductive duct naturally produces free radicals through metabolism process. Under certain conditions, in the body, free radicals are formed a lot more than antioxidant which works as free-radical neutralizer by binding the free-radicals.

Reactive oxygen species (ROS) is free radicals in the form of oxygen derivative (Agarwal et al. 2003). Reactive oxygen species can cause damage to cell structure like mitochondria and microtubule. It also causes the disruption of cell function when the critical concentration of ROS is overwhelming (Roushandeh et al. 2008). Thus, influence the qualities of oocyte, spermatozoa, embryo and also the media culture (Agarwal et al. 2005). Cell physiological functions must be kept normal by constant inactivation of ROS or by keeping free radicals at the low number by giving antioxidants such as tocopherol (vitamin E) and beta-carotene (vitamin A) (Sarungallo et al.2015).

Red fruit (*Pandanus conoideus* Lam) is an endemic fruit from Papua and has been used by local people as a natural antioxidant. According to Sarungallo et al (2015) *P. conoideus* contains a natural antioxidant indicated by the high level of α-tocopherol and β-carotene. Alpha-tocopherol and β-carotene are antioxidant compounds that can bind to free radicals (Wojcik et al. 2010). Alpha-tocopherol (vitamin E) is known as a secondary antioxidant that prevents the forming of free radicals, inactivate oxygen singlet and prevents lipid peroxidation on the plasma membrane (Rajalakshmi and Narasimhan 1996). β-carotene is a pigment isoprenoid in plants that can destruct and inactivate ROS and protects cells from oxidative damage (Bendich and Saphiro 1986). The study conducted by Chen et al (2011) reported that *P. conoideus* contains both antioxidant compounds, each 700 ppm of α-tocopherol and 500 ppm of β-carotene (Schierle et al. 2003). The antioxidant compounds in *P. conoideus* are expected to bind to ROS found in the female reproductive system.

Therefore, the objective of this research was to observe the influence of *P. conoideus* (ERF) extract given to mice (in vivo) on the quality of oocyte, fertility and in vitro early development of mice embryo.

MATERIALS AND METHODS

The extraction of *Pandanus conoideus*

The method used to extract the red fruit as follow, the red fruit flesh was cut in pieces, rinsed in clean water, steamed for 1-2 hours until tender and then let it cool. The flesh of the fruit was pulverized by hand, put in a sieve to separate its seeds. The clean and smooth pulverized fruit were then cooked on medium flame (40^{o}C) for 5-6 hours, stirred throughout. Then took from the stove and let it for one day until three layers were formed; dregs (at the bottom), water (in the middle) and oil (at the top). Next, the oil was taken and used for this research.

Treatment to mice

The samples used were 40 female mice divided into three types of treatments. Each treatment used fifteen mice with three replications (Table 1). Red fruit extract is given to female mice orally for seven days in a row before superovulation (in vivo) with the dosage used was based on Sakinah (2007).

Superovulation

Female mice of 3-4 months (weighing around 23.2 grams) were superovulated by injecting 5 IU PMSG, followed by injection of 5 IU hCG each, *intraperitoneally*. The injection of hCG was done 48 hours after the injection of PMSG (Hogan et al. 1986).

Sperm preparation

Cauda epididymis was collected by killing male mice *dislokasio os cervicalis* and then rinsed in CZB collecting media. *Cauda epididymis* was placed in a petri dish which was added 500 µl CZB collecting media. *Cauda epididymis* was sliced to get sperm using 21 *gauge* sterile needle until sperm was released from *cauda epididymis*. Sperm was incubated in a CO_2 in at 37^{o}C (Thermo scientific, 381, USA) for one hour.

Oocytes collecting

The superovulated female mice were killed by *dislokasio os cervicalis*. Oocyte collecting was done by making a slice on the oviduct using 21 *gauge* sterile needles. The slicing was done in CZB collecting media + *hyaluronidase* coated with *mineral oil* of 50 µl. Oocyte taken from oviduct was placed into CZB rinsing media 50 µl without *hyaluronidase* coated with *mineral oil.* Oocyte was cleaned from cumulus cells by pipetting. The already clean oocyte was then put into a small petri dish containing CZB fertilization media of 50 µl for each drop that was covered with *mineral oil*. Each drop contained 20-25 oocytes.

In vitro fertilization

Media used for in vitro fertilization was CZB without glucose and contained *Bovine Serum Albumin* (BSA) (Sigma, A3311, St. Louis, MO, USA) 1mg/mL. Media was covered with *mineral oil* (Sigma, M8410, St. Lois, MO, USA) and stored in a CO_2 incubator (Thermo scientific, 381, USA) at 37^{o}C. In vitro fertilization was done by putting sperm into drops containing oocytes. Sperm was added using a micro pipet as much as 50 µl (concentration 2 x 10^{6}) and incubated for 4 hours in a CO_2 incubator at 37^{o}C (Thermo scientific, 381, USA).

In vitro culture

In vitro culture was complete after 4 hours of incubation. The zygote from the culture was examined and transferred to the CZB culture media. The media then added 5.55 mM D-glucose (Merck, 1.08342.0500, Darmstadt, Germany) and 1 mg/mL *Bovine Serum Albumin* (BSA) (Sigma, A3311, St. Louis, MO, USA), then 50µl was dropped in a petri dish. The media was covered with *mineral oil* (Sigma, M8410, St. Lois, MO, USA) and incubated in a CO2 incubator at a temperature of 37°C (Thermo scientific, 381, USA). The observation was done 24 hours later. The media was renewed every 48 hours.

Parameters

Parameters observed in this study were the quality of oocytes, the fertility rate of mice that were given the extract of red fruit (ERF) and early embryos development rate. The quality of oocytes was measured by looking at the quality whether it was grade A (oocytes were surrounded by a layer of evenly grown cumulus oophorus), grade B (oocytes were surrounded by an uneven layer of cumulus oophorus), and grade C (oocytes without oophorus cumulus). The parameter for mice fertility level was measured by looking at the percentage of pronucleus (PN) formed; i.e. 1 PN and 2 PN. The parameter for early embryo development was determined by number the percentage of embryo development (2 cells, 4-8 cells, and morula-blastocyst).

Experimental design

This study had Completely Randomized Design (CRD) with three treatments and three replications. Parameter taken were the quality of oocytes, fertility rate and in vitro early embryo development. The sample used in this study was mice. Every treatment consisted of ERF concentration for each 0 mL ERF, 0.05 mL ERF, and 0.1 mL ERF.

Data analysis

Data was collected quantitatively to all parameters. When data were normally distributed, differences between treatment groups were assessed by one-way analysis of variance (ANOVA) based on F test phase 5%, and if there is a real impact, it will be continued with Duncan test phase 5%. The data was analyzed using software SPSS 17.

RESULTS AND DISCUSSION

Oocytes quality

The quality of oocytes in this research was determined by cumulus cells surrounding oocytes, compact and homogenous cytoplasmic. According to Susilowati et al. (1998) the quality of mammal oocytes can be divided based on three criteria which are *grade* A oocytes surrounded by 7-5 layers of oophorus cumulus, *grade* B oocytes surrounded by an uneven layer of oophorus cumulus and *grade* C oocytes without oophorus cumulus. The result of observation on oocytes quality (Table 2). Table 2 showed that there were no significant differences (p>0.05) between each treatment on the quality of oocytes at grade A, B and C. But, the oocyte quality in grade A was higher than grade B and C.

Fertility level

The rate of oocytes fertilization in this research can be seen from the percentage of pronucleus formed. The result of statistic test done to the percentage of pronucleus formed, for fertility rate (Table 3).

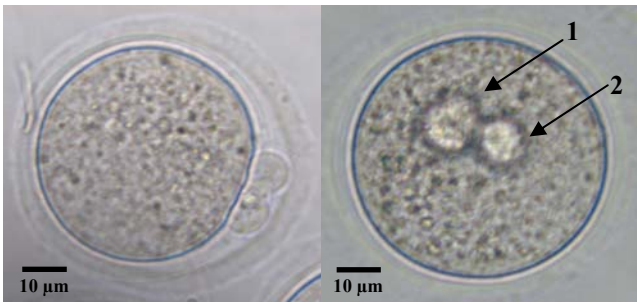
In this research, extract *P. conoideus* supplementing did not give real effect on in vitro fertilization rate. The image of the forming of 2 pronucleus can be seen in Figure 1.

The supplementation of ERF for fertility level with pronucleus forming is not different significanly (p>0.05) between control treatment, 0.05 mL Extract *P. conoideus* and 0.1 mL ERF consecutively i.e. (58.85 ± 17.12[a]), (46.64 ± 7.28[a]), (59.22 ± 16.30[a]).

Embryo development during pre-implantation stage

The effect of supplementing ERF on early embryo development seen from the percentage of the forming of 2 cells, 4-8 cells, morula-blastocyst (Figure 2). The results of ANOVA test on early embryo development are shown in Table 4.

The ERF supplementation has no significant difference (p>0.05) on early embryo development at every treatment (control is 25.00 ± 3.60[a], 0.05 mL ERF is 44.44 ± 1.15[a], and 0.1 mL ERF 76.67 ± 6.81[a]). The development of two cell is slightly different from the development of four-eight cells and morula-blastocyst.



A B

Figure 1. A. Oocyte before fertilized, B. Oocyte with 2PN. Note: 1. Male pronucleus; 2. Female pronucleus

Table 2. The effect of Extract *Pandanus conoideus* supplement on the quality of oocytes

Treatment	Number of oocytes examined	Quality of oocytes (% ± SD)		
		A	B	C
Control	363	45.01[a] ± 34.65	26.99[b] ± 0.00	27.99[b] ± 3.05
0.05 mL ERF	215	48.33[a] ± 27.46	27.50[b] ± 5.00	24.16[b] ± 6.42
0.1 mL ERF	333	68.39[a] ± 0.00	23.99[b] ± 14.29	7.62[b] ± 3.05

Note: Values with different supersripts in the same letter in the same column differ significantly (P<0.05, Duncan)

Table 3. The effect of Extract *Pandanus conoideus* supplement on in vitro fertilization rate

Treatment	Number of oocytes	Pronucleus (PN) formed (% ± SD)	
		1 PN	2 PN
Control	272	41.17 ± 17.17[a]	58.85 ± 17.12[a]
0.05 mL ERF	166	52.60 ± 8.09[a]	46.64 ± 7.28[a]
0.1 mL ERF	311	41.06 ± 16.68[a]	59.22 ± 16.30[a]

Note: Values with different supersripts in the same letter in the same column differ significantly (P<0.05, Duncan)

Table 4. The effect of supplementing ERF on early embryo development in vitro

Treatment	Number of oocytes examined	Number (%) of oocytes which development (% ± SD)		
		2 Cells	4-8 Cells	Morula-blastocyst
Control	143	60(41.96 ± 29.46)[b]	48(80.00 ± 24.33)[ab]	12 (25.00 ± 3.60)[a]
0.05 mL ERF	79	63(79.75 ± 23.30)[b]	9 (14.29 ± 3.61) [ab]	4 (4.,44 ± 1.15)[a]
0.1 mL ERF	196	98(50.00 ± 30.50)[b]	30 (30.61 ± 11.14) [ab]	23 (76.67 ± 6.81)[a]

Note: Values with different supersripts in the same letter in the same column differ significantly (P<0.05, Duncan)

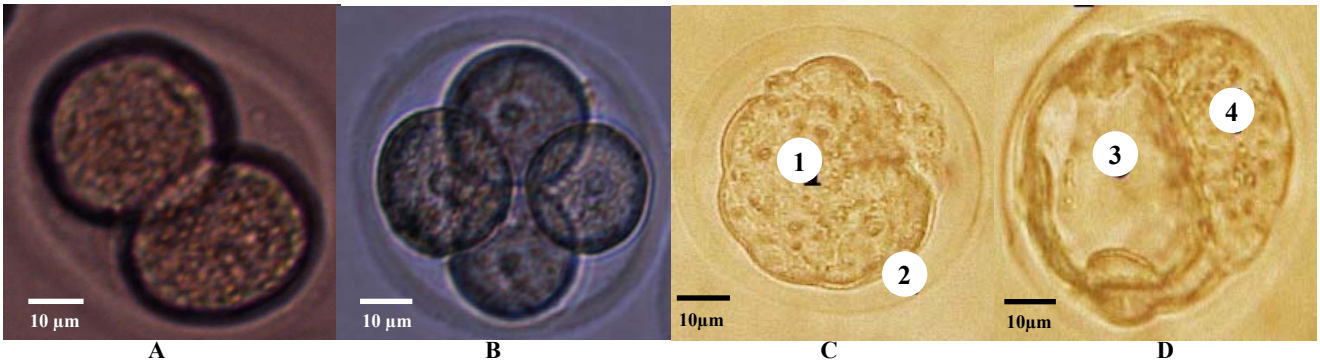


Figure 2. Zygote cleavage into: A. 2 cells, B. 4 cells, C. morula, D. blastocyst. Note: 1. *blastomer cell*; 2. *pelucida* zone ; 3. *blastocoel*; 4. *inner cell mass*

Discussion

Quality of oocytes

The supplementation of ERF in this research did not have any effect on the quality of oocytes. Extract red fruit was reported to have antioxidant and effect on the female reproductive environment (Agarwal and Saleh 2002; Maslachah et al. 2004). But the dosage of ERF used in this research was not sufficient to increase the quality of oocytes nor protect oocytes from free radicals that were formed during metabolism process. The unstable condition of culture media made it easy to form free radicals produced by reactive oxygen species (ROS). According to De Matos (2002), oocytes in culture media still had Glutathione Sulfur Hydroxyl (GSH) in high number. GSH level in this research and an addition α-tocopherol and β-carotene were presumed to be not sufficient to tolerate the forming of free radicals formed during fertilization process. Furthermore, it was assumed that there was a decline in GSH level due to a negative reaction between GSH and antioxidant (α-tocopherol and β-carotene). And further research on GSH level in oocytes after supplementing antioxidant is needed.

Pandanus conoideus extracts given to female mice for seven days in this research did not give a positive effect on female reproductive system environment. Reactive oxygen species produced from metabolism remnants in the body could give effect to the quality of oocytes located in the female reproductive duct. The oocyte is a functional cell where change regulation takes place, like filtering energy from the environment, reacting to the environment and developing by doing metabolism (Fleming et al. 2004).

The compound antioxidant used in this study could not create an environment for a female reproductive system that reduces oxidation reaction in metabolism process in the female of the tract. It is assumed that it could not increase GSH level in the oocyte, and thus could not support oocyte maturation process nor minimize the forming of ROS during in vitro fertilization.

Supplementing 0.1 mL ($68.39^{a} \pm 0.00$) ERF in this study did not has significant effect on the improvement of the quality of oocyte and fertility level which was marked by the forming of two pronucleus. It is in accordance with the study of Yasmin et al. (2015) who stated that administering antioxidant (*cericin*) to oocyte could not support maturation process. According to Gordon (2003), the maturation level of oocyte nucleus was an indicator of the development of oocyte competence. During maturation, there is continuance from meiosis process which is initiated by *vesicle breakdown* (GVBD) after *germinal vesicle* (GV) stage.

Fertilization rate

Supplementing ERF as an antioxidant to mice was an effort to improve the quality of oocyte which in return will affect the success of fertilization. But, in this research, supplementing ERF could not increase the quality of oocyte which in turn did not influence fertilization rate. The percentage of pronucleus formed was taken as two pronuclei formed; which was 4 hours after fertilization (Ayu, 2005). Male pronucleus came first into form than the female one (Harjanti 2002; Ayu 2005). The size of male pronucleus is usually bigger than female pronucleus (Rugh 1968; Hafez 1970; Ayu 2005) (Figure 1).

The evaluation of the success of fertilization in this research is measured by the number of pronucleus formed with the criteria of monosperm (2 PN). In this research α-tocopherol and β-carotene were given to mice to evaluate their potential as antioxidants, but no significant difference was found between control and treatment. The treatment of supplementing ERF did not have any effect on the forming of pronucleus or fertility rate. Maybe the unstable culture condition increased the number of free radicals. The mastering of technique might also be the reason for the small success in this research.

Furthermore, the high frequency of *polysperm* could also increase *gamma glutamyl transpeptidase*, and thus decreased GSH in the oocyte. The decline of GSH in oocyte detained the forming of male pronucleus.

α-tocopherol and β-*carotene* contained in the extract of *P. conoideus* (ERF) can act as an antioxidant which will neutralize and inactivate or stabilize ROS. *α-tocopherol* and β-*carotene* are reported to have antioxidant activities and prevent cell damage due to free radicals through detaining lipid peroxide (Kato et al. 1998). But α-*tocopherol* and β-*carotene* in this research had no effect, probably because the dosage given was not sufficient to

chelate lipid peroxidation. Hydroxyl group in amino acid detains lipid peroxidation by binding with ROS (Chlapanidas et al. 2013), and so becomes a more stable compound and also by binding reactive metal such as iron (Fe^{2+}) and copper (Cu^{2+}) which triggers lipid peroxidation (Patel and Modasiya 2011; Chlapanidas et al. 2013). The transition of metal ions like Fe2+ and Cu2+ catalyzes the production of dangerous free radicals (Halliwell and Gutteridge 1990). According to Minnoti and August (1989), Fe2+ is the most detrimental to the cell, Fe2+ can induce anion superoxidation to become hydroxyl radicals. Young and Woodside (2001) reported that the production of hydroxyl radicals catalyzed by Fe2+ would oxidize lipid and cause oxidative stress. The binding of reactive metal by amino hydroxyl will reduce metal reactivity and also prevent reaction producing hydroxyl radicals.

It is assumed that supplementing ERF with treatment 0.1 mL (59.22 ± 16.30^a) and 0.05 mL (46.64 ± 7.28^a) did not affect fertilization level because of the unstable fertilization media so that during fertilization process, oocyte could not ward off free radicals which were formed during fertilization process. The remnants of spermatozoa metabolism could also become a factor that detained the forming of two pronuclei. It happened because the preserved sperm released oxygen derivate as metabolism remnant.

In this research, oocyte was not capable of detaining free radicals produced by sperm. It could not support sperm capacitating process and continue acrosomal reaction so that male pronucleus could not form. The result of this research showed that there was no significant difference between supplementing ERF with and without, 0.1 mL (59.22 ± 16.30^a), 0.05 mL (46.64 ± 7.28^a) and control (58.85 ± 17.12^a). But the treatment of 0.05 mL gave lower result than control. It could be because the treatment had a negative impact and toxic so that *reactive oxygen species* (ROS) formed during in vitro fertilization process was not prevented by antioxidant.

Sperm used for fertilization had been preserved in a CO_2 5% incubator for one hour. During preservation, it is assumed that sperm motility declines because of metabolism remnant. It can detain fertilization and the forming of *pronucleus* because of the overwhelming of free radicals produced by sperm. According to Eriani et al. (2008a) sperm is still alive during preservation process and keeps its activity by utilizing energy from metabolism. But this energy will run out and lactic acid as metabolism by-product increases. Lactate acid can decrease pH which is toxic and has the potential to damage enzymes needed for metabolism, and thus disturbs metabolism. Soeradi (2004) said that the decline of sperm motility was also caused by ROS. ROS can induce damage to DNA (Fraser, 2004). It is backed up by Suryodhono (2000) who said radicals such as hydroxyl (OH) and oxygen singlet (O_2^-) could damage three important compounds (fatty acid, DNA, and protein) needed to maintain cell integrity.

Failure in fertilization can be caused by several cases, among others, insufficient oocyte maturation (nucleus and cytoplasmic level), insufficient sperm capacity to fertilize oocyte (capacitating and acrosome reaction), sperm condensate inside oocyte cytoplasmic, so it fails to form male *pronucleus* in fertilization can be caused by several cases, among others, insufficient oocyte (nucleus and cytoplasmic), insufficient sperm capacity to fertilize oocyte (capacitating and acrosome reaction), sperm condensate inside oocyte cytoplasmic so it fails to form male *pronucleus* (Boediono 2001).

According to Harjanti (2002), there are two types of abnormality happened in the fertilization process. First, spermatozoa fail to penetrate oocyte and *decondensation* sperm is detained. The second type, there are two or more *pronucleus* as result of penetration from more than one spermatozoa and causes polyploidy in a zygote. The quality of oocyte influences *polysperm*. Boediono (2001) added that oocyte's age could decrease zone's reaction ability to ward off *polysperm* that penetrate zone and *vitellin*. Not only influenced by the quality of oocyte, but *polysperm* is also influenced by spermatozoa concentration.

Early embryo development

This research showed that embryo obtained from in vitro fertilization from mice that had been treated with ERF grew to morula and blastocyst stage. There was no significant influence ($p>0.05$) on embryo development between control, 0.1 mL ERF and 0.05 mL ERF consecutively (25.00 ± 3.60^a), (44.44 ± 1.15^a), and (76.67 ± 6.81^a). But seen from the percentage, ERF dosage 0.1 mL was the highest that reached morula and blastocyst phase. Isobe et al. (2012) reported that alpha-tocopherol as an antioxidant has the potential to enhance embryo cell proliferation so that the percentage of produced blastocyst increases. In this study, α-*tocopherol* and β-*carotene* were in vivo supplemented, to evaluate the potential of ERF's antioxidant. In this research, the embryo was able to cleavage to morula and blastocyst stage. Supplementing 0.1 mL ERF in vitro had no significant difference ($p>0.05$) compared to control and 0.05 mL ERF.

The treatment given to mice was assumed to be insufficient for embryo development. Study on the influence of supplementing vitamin E on mice's fertility, cleavage, and embryo development had been conducted by Hassa et al. (2007), but the result showed there was no influence. Further, a study was done by Jishage et al. (2005) on mice, showed that α-tocopherol was needed for proliferation or the functioning of a placenta.

The absence of significant difference between treatment with and without treatment is assumed because of the inability of cleavage in detaining free radicals. Another factor that might have detained embryo development was the media used for embryo culture. The media used in this research was CZB.

Several embryos were behind in development and did not reach morula stage. It was assumed that glucose concentration in in vitro culture media was not fit for every stage of embryo development. Embryo obtained from in vitro fertilization or being cultured in zygote stage in culture media will be detained during the early embryo development, also known as cell-block (Djuwita et al. 2000; Eriani et al. 2008b). It is common to find cell-block in embryos from many species which is cultured in vitro.

Mice and rat embryos are often found two cell-block. It is greatly influenced by glucose concentration in in vitro culture media. Two-cell block causes the obstacle of some embryos to the next stage development so that not all embryos in in vitro culture can develop into morula.

The high level of glucose will block embryos at two-cell stage. It is assumed that the high level of glucose produces more free radicals compared to the amount of pyruvate that can bind them, and so, disturb early embryo development (Hafez 2000). Garner and Leese (1990) also reported that energy gained from glucose could not be utilized optimally because glucose cannot be transported to mitochondria during early division due to decline in glycolysis because of negative feedback that detained phosphofructokinase enzyme.

ACKNOWLEDGEMENTS

Authors wish to thank Research Center for Biotechnology, Indonesian Institute of Sciences who had allowed authors to conduct research.

REFERENCES

Agarwal A, Prabakaran SA. 2005. Oxidative Stress and Antioxidan in Male Infertility. Iranian J Reprod Med 3(1): 1-8.

Agarwal A, Saleh RA. 2002. Review article: Role of oxidants in male infertility: Rationale, significance, and treatment. Urol Clin North Amer 29: 1-11.

Agarwal A, Saleh RA, Bedaiwy MA. 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 79: 829-843.

Ayu DR. 2005. The formation of pronucleus male and female after fertilization on mice (*Mus musculus*). [Thesis]. Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta. [Indonesian]

Bendich A, Shapiro SS. 1986. Effect of β-carotene and canthaxanthin on the immune response of the rat. J Nutr. 116: 2254-2262.

Boediono A. 2001. Sperm Immobilization Prior to Intracytoplasmic Super Injection (ICSI) and oosyte activation improves early development of microfertilized goat. Reprotech 1 (1): 29-34.

Chen, H., Angiuli, M., CFerrari, C., Tombari, E., Salvetti, G. and Bramanti, E. 2011. Tocopherol speciation as first screening for the assessment of extra virgin olive oil quality by reversed-phase high-performance liquid chromatography/fluorescence detector. Food Chemistry 125(4): 1423-1429

Chlapanidas T, Farago S, Lucconi G, Pertehella S, Galuzzi M, Manteli M, Avanzini MA, Tosca MC, Marazzi M, Vigo D, Torre ML, Faustini M. 2013. Sericins exhibit ROS-scavengin m.g, anti-tyrosinase, anti-elastase and in vitro immunomodulatry activities. Int J Biol Macromol 58: 47-56.

De-Matos DG, Gasparrini B, Pasqualini SR, Thompson JG. 2002. Effect of glutathione synthesis stimulation during in vitro maturation of ovine oocytes on embryo development and intracelluler peroxide content. Theriogenology 57: 1443-1451.

Djuwita, I, Amalia L, Widjiati, Mohamad K. 2000. Effect of glucose concentration in the medium with and without phosphates on the development of preimplantation mouse embryos in vitro. Med Vet 7 (1): 9-12. [Indonesian]

Eriani K, Boediono A, Djuwita I. Sumarsono HS, Azhar A. 2008a. Development of domestic cat embryo produced by preserved sperms. Hayati J Biosci 15 (4): 155-160.

Eriani K. Sunarti. Nasir M, Djuwita I. 2008b. The influence of serum-free culture medium on the embryonic development of mice in vitro. Kedokteran Yarsi. 16 (1): 28-32. [Indonesian]

Fleming TP, Kwong WY, Porter R, Ursell E, Fesenko I, Wilkins A, Miller DJ, Watkins AJ, Eckert JJ. 2004. The embryo and its future. Biol Reprod. 71: 1046-1054.

Fraser LR. 2004. Structural Damage to Nuclear DNA in Mammalian Spermatozoa: its evaluation techniques and relationship with male infertility. Polish J Vet Sci 7 (4): 311-321.

Garner DL. Hafez ESE. 2000. Spermatozoa and seminal plasma. Reprod Farm Anim 7: 96-109.

Gardner DK, Leese HJ. 1990. Concentration of nutrients in mouse oviduct fluid and their effects of embryos development and metabolism in vitro. Reprod Fertil 104: 91-97.

Gordon I. 1994. Laboratory Production of Cattle Embryos. Biotechnology in Agriculture Series. CAB International.

Gordon I. 2003. Laboratory Production of Cattle Embryos. Ed ke-2. London (GB): CABI Publishing.

Hafez ESE. Hafez B. 2000. Fertilization and cleavage. Reprod Farm Anim 7: 117.

Harjanti DW. 2002. The Development of Nuclei from Ovum and Sperm Cells Post-Fertilization of In Vivo and In Vitro in Mice (*Mus musculus albinus*). [Hon. Thesis]. Faculty of Veterinary Medicine, Institut Pertanian Bogor, Bogor. [Indonesian]

Hassa H, Gurer F, Tanir HM, Kaya MNB, Gunduz A, Sariboyaci E, Bal C. 2007. Effect of Cigarette Smoke and Alpha-Tocopherol (Vitamin E) on fertilization, cleavage, and embryo development rates in mice: an experimental in vitro fertilization mice model study. Eur J Obstet Gynecol Reprod Biol 15: 1802.

Hoshi M, Toyoda Y. 1985. Effect of EDTA on the Preimplantation Development of Mouse Embryos Fertilized In vitro. Jpn J Zootech Sci 56: 931-937.

Hogan B, Beddington R, Costantini F, Lacy E. 1986. Manipulating the mouse embryo laboratory manual. Cold Spring Harbor Laboratory Press, Danvers.

Isobe T, Ikebata Y, Onitsuka T, Wittayarat M, Sato Y, Taniguchi M, Otoi T. 2012. Effect of Sericin on Preimplantation Development of Bovine Embryos Cultured Individually. Theriogenology 78: 747-752.

Jishage K, Tachibe T, Ito T, Shibata N, Suzuki S, Mori T, Hani T, Arai H, Suzuki H. 2005. Vitamin E is Essential for Mouse Placentation but Not for Embryonic Development it Self. Biol Reprod 73: 983-987.

Kato N, Sato S, Yamanaka A, Yamada H, Fuwa N, Nomura M. 1998. Silk protein sericin, inhibits lipid peroxidation and tyrosinase activity. Biosci Biochem 62: 145-147.

Maslachah L, Sukmadi M, Sugihartut R. 2004. The effect of alpha-tocopherol on spermatogenesis of mice that received stressors. J Pend Med Eks 5: 258-269. [Indonesian]

Minotti G, August SD. 1989. The role of iron in oxygen radicals mediated lipid peroxidation. Chem. Biol Interact 71: 1-9.

Patel RJ, Modasiya MK. 2011. Sericin: Pharmaceutical application. Int J Res Biomed Sci 54: 132-139.

Roushandeh AM, Pasbakhsh P, Alizadeh Z, Roudkenar MH. 2008. In vitro maturation media cysteamine concentration and glutathion level affect blastocysts development in mouse. Iranian J Reprod Med 5(4).

Rugh R. 1989. The Mouse its Reproduction and Development. Burgess Publishing Company, USA.

Sakinah N, Sukrasno, Immaculata M. 2007. The effects of Ethanol Extracts Sambiloto (*Andrographis paniculata* Ness.) and Red Fruit Oil (*Pandanus conoideus* Lam.) on Immune Response of Mice. School of Pharmacy, Institut Technology Bandung, Bandung. [Indonesian]

Sarungallo ZL, Hariyadia P, Andarwulana N, Purnomoa EH. 2015. Analysis of α-cryptoxanthin, β-cryptoxanthin, α-carotene, and β-carotene of Pandanus conoideus oil by high-performance liquid chromatography (HPLC). Procedia Food Science 3: 231 – 243

Schierle, J., Schellenberger, T., Fizet, C. and Betz , R. 2003. A simple spectrophotometric determination of total β-carotene in food additives with varying E/Zisomer ratios using an isobestic wavelength. European Food Research and Technology 215:268–274

Soeradi O. 2004. Free radicals in infertil men. Proceeding Paradigm Genetics and Reproduction. Departmentt of Biology, Faculty of Medicine, Universitas Indonesia, Jakarta.

Suryodhono. 2000. Oxidants, Antioxidants and Free Radicals. CV Sagung Seto, Jakarta. [Indonesian]

Susilowati T, Sumitro SB, Djati MS, Ciptadi G, Permono B. 1998. Optimization of oocyte maturation in vitro by a combination of serum and hormone concentrations in TCM 199. Natural J 2 (1): 16-23. [Indonesian]

Wojcik M, Burzynska, Pedziwiatr I, Wozniak LA. 2010. A review of natural and synthetic antioxidants important for health and longevity. Curr Med Chem 17: 3262-3288.

Yasmin C, Otoi T, Setiadi M, Karja NWK. 2015. Maturation and fertilisation of sheep oocytes cultured in serum-free medium containing silk protein sericin. Acta Vet Hungarica 63 (1): 110-117.

Young IS, Woodside JV. 2001. Antioxidant in health and disease. J Clin Pathol 54:176-186.