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INFLUENCES OF INCUBATION TIME AND SUCROSE CONCENTRATION ON MICE (*Mus musculus* L.) OOCYTE VIABILITY FOR ENUCLEATING PROCEDURE

Ekayanti Mulyawati Kaiin[1]*, Muhammad Gunawan[1], Ghina Nafisah[2], and Madihah Madihah[2]
[1]Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Jakarta, Indonesia
[2]Biology Department Faculty of Mathematics and Natural Science, Padjajaran University, Bandung, Indonesia
*Corresponding author: ekayantimk@yahoo.com

ABSTRACT

This study aimed to find out the optimum incubation time to complete mouse oocyte maturation at Metaphase II (MII) stage and determine the optimum sucrose concentration enabling to induce nuclear swelling for visualization that is important for enucleating process at the initial procedure of somatic cell nuclear transfer (SCNT). In this current study, mice were used as animal model. Completely randomized design was arranged, consists of 2 trials with 4 treatments and 7 replications. In the first trial, the oocytes were cultured at 0-2, 4-6, 8-10, and 12-14 h in 5% CO_2 incubator at 37° C. Second, the MII oocytes obtained from previous trial were cultured in M199 medium containing different concentrations of sucrose (0, 1.5, 3, and 6%). The parameters measured were the oocyte viability at various stages, i.e germinal vesicle (GV), metaphase I (MI), anaphase/telophase I (A/T I), and metaphase II (MII), and the viability of swollen nuclear oocytes using Hoechst/PI staining. The results showed that the optimum incubation time required by oocytes to reach MII stage was 12-14 h with a percentage of 57.14±12.67%, while the optimum sucrose concentration for nuclear swelling was found at 3% with a percentage of 100±0.00%. Our findings provided preliminary results related to the maturation process of the mouse oocyte nucleus, which is meaningful for the initial procedure of SCNT.

Key words: metaphase II, mice, nuclear swelling, oocyte, SCNT

ABSTRAK

Tujuan penelitian ini adalah untuk mendapatkan waktu inkubasi optimum yang diperlukan oosit mencit agar mencapai tahapan metafase II dan konsentrasi optimum sukrosa agar inti oosit membengkak sehingga memudahkan visualisasi guna keperluan enukleasi pada prosedur awal proses teknik transfer inti sel somatik (TISS). Pada penelitian ini digunakan mencit sebagai hewan model pengembangan teknik TISS di Indonesia. Metode yang digunakan adalah eksperimental dengan rancangan acak lengkap (RAL), terdiri atas 2 tahap uji dan masing-masing 4 perlakuan dengan 7 pengulangan. Perlakuan pertama dilakukan dengan mengkultur oosit selama 0-2, 4-6, 8-10, atau 12-14 jam di dalam inkubator CO_2 5% dengan suhu 37° C. Perlakuan kedua dilakukan dengan mengkultur oosit tahap metafase II dari hasil perlakuan pertama dalam medium M199 dengan berbagai konsentrasi sukrosa yaitu 0; 1,5; 3, dan 6%. Parameter yang diamati adalah persentase oosit yang hidup pascainkubasi pada berbagai tahap perkembangan yaitu vesikula germinalis (VG), metafase I (MI), anafase/telofase I (A/T I), dan metafase II (MII), serta persentase oosit hidup dengan inti membengkak yang diamati dengan menggunakan pewarnaan Hoechst/PI. Hasil pengamatan menunjukkan bahwa waktu kultur optimum agar oosit mencit mencapai tahapan metafase II adalah 12-14 jam dengan persentase sebesar 57,14±12,67% dan konsentrasi optimum sukrosa agar inti oosit membengkak adalah 3% dengan persentase sebesar 100±0,00%. Penelitian ini dapat dijadikan informasi awal mengenai proses pematangan inti oosit mencit yang dapat diaplikasikan dalam prosedur awal TISS.

Kata kunci: metafase II, mencit, pembengkakan inti, oosit, TISS

INTRODUCTION

Somatic cell nuclear transfer (SCNT) constitutes one of the assisted reproductive technologies which could be used for species conservation. The SCNT is able to conserve the desirable characteristics of animals; thus, it can be applied in reproduction process (Sumer and Paul, 2011). Previously, sheep was successfully cloned using SCNT technique (Campbell *et al.,* 1996). The initial stage of SCNT is to induce mature oocyte in which the nucleus was then replaced by somatic cell nucleus. This initial step was crucial and could determine the result of further steps, namely transferring somatic cell nucleus into the enucleated oocytes (Wun and Ralph, 2008).

The duration of oocyte maturation in some species may vary, even in the same phylogenetic class (Masui and Clarke, 1979), including mammalian such as 24 h for lamb (Moor and Trounson, 1977), 23-35 h for goat (Song and Iritani, 1987), and 6-10 h for rabbit (Chang, 1955; 1995). The oocyte reached maturity at metaphase II (MII) stage in which the nucleus was ready for fertilization. The observation of MII oocyte nucleus could be improved by incorporation of sucrose in medium. The oocyte nucleus become swelling due to the presence of sucrose, which contributed to easily visualize its spindle and chromosome (Wang *et al*., 2001a). This current work investigates MII enucleated oocyte on mice as recipient for somatic nucleus transfer previously reported by Kono *et al.* (1993) using M2 medium, while study pertaining in vitro maturation of mouse oocyte nucleus was carried out by Fulka *et al*. (1995). In Indonesia, scientific reports on SCNT for both livestock and animal model a are rather scarce. This present work aimed to observe the optimum incubation time for maturation of MII oocyte and determine the optimum concentration of sucrose enabling to swell mouse oocyte nucleus; thus, we could easily visualize the nuclear component for enucleating procedure in SCNT.

MATERIALS AND METHODS

Superovulation

Female mice strain DDY (Deutschland Denken and Yoken) were used as animal model and obtained from

Faculty of Veterinary Medicine, Bogor Agricultural University. Female mice (age of 8-10 weeks, average weight of 40-45 g) were given 5 IU of *pregnant mare's serum gonadotropin* (PMSG) hormone per mouse by intraperitoneally (i.p), followed by i.p injection of human chorionic gonadotropin (hCG) (5 IU per mouse) after 48 h (Hogan *et al*., 1986). The mice were then reared for 15-16 h and fed ad libitum (Heidari *et al*., 2012).

Collection, Culture, and Staining

After 15-16 h of hCG injection, mice were anesthetized and sacrificed. The reproductive tracts (ovarium, oviduct, and uterus) of the mice were collected. The oviduct was separated from ovary and uterus, and stored in DPBS collection medium containing gentamycin 50 mg/mL and 1% bovine serum (BS). Syringe 1 mL and dissecting needle 26 G were used to tear open the ampulla of the oviduct and release oocyte. The oocyte was then transferred using holding pipettes into 35 mm petri dish (Corning) containing collection medium, washed again by the collection medium three times to discard unwanted materials. The washed oocyte was stored in CZB medium (Gunawan *et al*., 2014) for 1-3 min to remove cumulus cells (Polanski, 1986) and washed again by collection medium to remove remaining cumulus cells (Wang *et al*., 2001b). The oocyte was stored in M199 medium in sterilized culture dish for further step.

Oocyte in M199 medium was incubated for different periods (0-2, 4-6, 8-10, and 12-14 h) in incubator at CO_2 level of 5% and temperature of 37° C. Afterward, the viability and growth of oocyte was observed under inverted microscope Diaphot 300 (Nikon), stained using Hoechst 33342-propidium iodide (Hoechst-PI) for 10-15 min and observed under fluorescence microscope Imager Z (Gunawan *et al.,* 2014). The observation was carried out to understand the growth stages of oocyte nucleus, namely germinal vesicle (GV), metaphase I (MI), anaphase/telophase I (A/T I), and metaphase II (MII) according to Hafez and Hafez (2000) with modification as presented in Table 1. The viable oocyte was marked as blue, while the dead oocyte was marked as red.

Visualization of Oocyte Nucleus using Sucrose-Containing Medium

The mature MII oocyte obtained from the first step was transferred into M199 medium contained various levels of sucrose (0, 1.5, 3, and 6%) to trigger swelling of oocyte nucleus. The swelling occurred around chromosome at MII stage, resulting in a transparent area around spindle like "∞" and "0" shape (Wang *et al*., 2001a). The viability and swelling of oocyte nucleus was observed with aid of Hoechst-PI staining under fluorescence microscope (Gunawan *et al.,* 2014).The observation of the viable oocyte was carried out after the treatment.

Analysis Data

Data were evaluated using analysis of variance (ANOVA) at 95%, while significant difference between means was compared using Duncan Multiple Range Test (Sudjana, 2012).

RESULTS AND DISCUSSION

Table 2 exhibits the effects of incubation time on the oocyte maturity of superovulated female mice after cultured in M199 medium. As presented in Table 2, incubation time at 0-2 h showed a greater number of MI oocyte (57.14%), while GV oocyte was found at 2.86 % and no MII oocyte was observed in this period of culture. Additionally, in a longer period of culture (4-6 h), the oocyte occurred in only two stages, i.e MI (40%) and A/T I (45.7%). However, in incubation time at 8-10 h, the oocyte at A/T I and MII stage reached 60% and 8.57%, respectively. Within 21-14 h, the number of MII oocyte showed a remarkable increase up to 57%.

Statistically, the number of GV oocyte was not significantly different in all incubation periods, while the number of MI oocyte in incubation period of 0-2 h

Table 1. Evaluation parameters of oocyte nucleus development

Classification	Description
Germinal Vesicle (GV)	Intact nucleus membrane, dense chromatin
Metaphase I (MI)	Homologous chromosomes arranged as double row at equatorial
Anaphase/Telophase (A/T)	Homologous chromosomes are separated to the opposite pole, chromosome become diffuse, nuclear membrane reform, and cytokinesis happened
Metaphase II (MII)	Sister chromatid align at equatorial and polar body I (PBI) was seen

Source: Hafez and Hafez (2000) with modification

Table 2. In vitro maturation of oocyte after different periods of culture (X±SD)

Time of maturation (hours)	Number of oocytes	Percentange of oocyte at stage (%)				Percentage of dead oocyte (%)
		GV	MI	A/T	MII	
0-2	35	2.86±2.86	57.14±6.80^{b}	8.56±4.04^{a}	0.00±0.00^{a}	28.57±7.37^{a}
4-6	35	0.00±0.00	40.00±7.56^{b}	45.7±7.20^{b}	0.00±0.00^{a}	14.28±5.71^{b}
8-10	35	0.00±0.00	20.00±8.73^{a}	60.00±11.55^{b}	8.57±5.95^{a}	11.43±5.94^{b}
12-14	35	0.00±0.00	2.86±2.86^{a}	34.30±8.41^{b}	57.14±12.67^{b}	5.71±5.71^{b}

$^{a, b}$Different superscripts within the same column indicate significant difference. GV= Germinal vesicle, MI = Metaphase I, A/T = Anaphase and telophase I, MII= Metaphase II

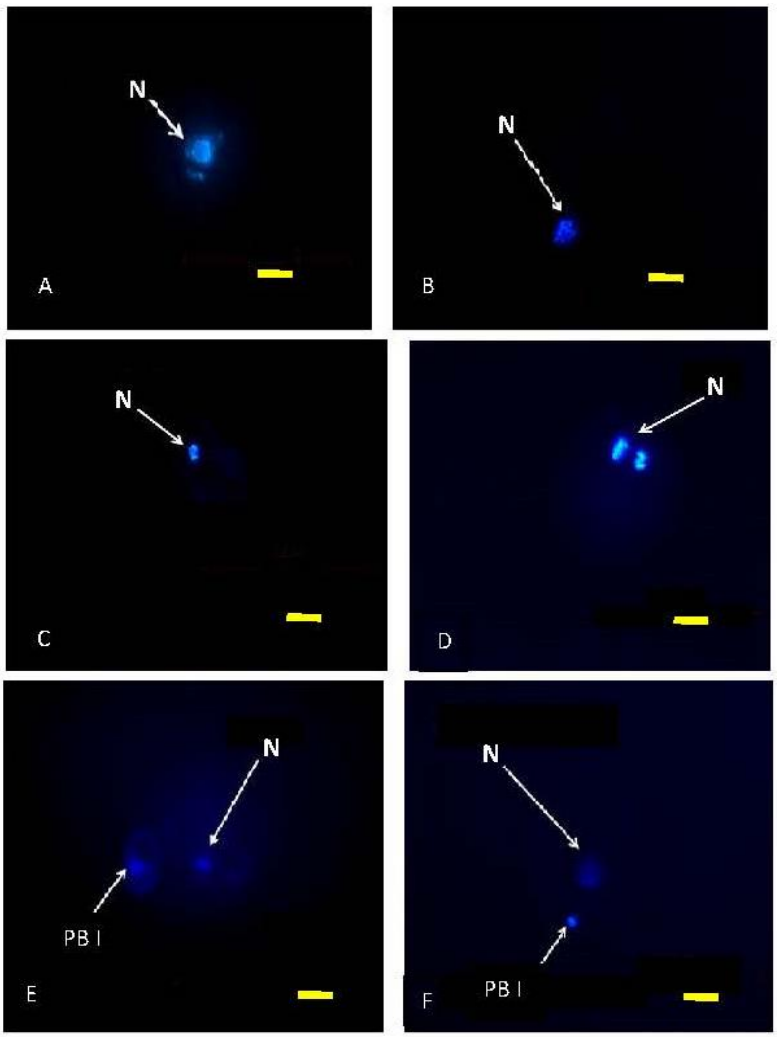

Figure 1. Microscopy images of oocyte at various stages. A= Germinal vesicle, B= Metaphase I, C= Anaphase I, D= Telophase I, E= Metaphase II, and F= Swollen nucleus oocyte, observed under fluorescense microscope at magnification of 400x and stained with Hoechst 33342-Propidium Iodide. PBI= Polar body I

and 4-6 h was significantly different compared with that in period of 8-10 h and 12-14 h ($P<0.05$). Furthermore, we also found that the number of oocyte at A/T I stage in incubation period of 0-2 h showed significant difference in comparison with that in incubation period of 4-6, 8-10, and 12-14 h ($P<0.05$). The MII oocyte in incubation period of 12-14 was significantly higher than in 0-2, 4-6, and 8-10 h ($P<0.05$). Hence, optimum attempts to reach mature and MII oocyte was found at incubation period of 12-14 h.

GV oocyte is depicted in Figure 1A which exhibits blue oocyte with intact nucleus membrane and dense chromatin. This result was in agreement with features of GV oocyte as reported by Hafez and Hafez (2000). Immature oocyte possesses a large nucleus called the germinal vesicle. The oocyte at the germinal vesicle stage underwent de-condensation, spreading, and transcription of most chromosomes. With the initiation of oocyte maturity, the transcription was inhibited, the chromosome was condensed, and nucleus membrane and nucleolus disappeared. Subsequently, the oocyte underwent germinal vesicle break down (GVBD) (Voronina and Wessel, 2003).

Figure 1B exhibits the MI oocyte in which the homologue chromosomes line up at the equator and appear blue in color. MI stage was indicated by arrangement of homologue chromosome in equatorial body and attachment of kinetochore from sister chromatid to microtubules in the opposite poles of the cell (Campbell *et al.*, 2000).

Oocyte at anaphase I stage is presented in Figure 1C, illustrating the separation of homologue chromosome to each pole. Anaphase I begins when the homologue chromosomes move toward opposite poles. This is because the microtubules attached in kinetochore of centromere start to shorten (Campbell *et al.*, 2000). Meanwhile, telophase I stage is shown in Figure 1D, illustrating that the spindle starts to disappear and two nuclei are formed through karyokinesis. In this stage, oocyte underwent nucleus reorganization and chromosome condensation to chromatin, followed by cytokinesis in which cytoplasm division into two cells occurred (Campbell *et al.*, 2000). Figure 1E exhibits oocyte at MII stage which is indicated by the presence of polar body I (PB I) and appeared blue in color. In this stage, sister chromatid attaches the microtubules in the spindle (Campbell *et al.*, 2000).

Table 3. The effect of sucrose on nucleus swelling at MII stage on mice (Mus musculus) oocyte (X±SD)

Sucrose concentration (%)	Number of oocyte at MII	Percentage of oocyte (%)	
		Swelling nucleus	Nucleus not swelling
0	20	16,67 ± 10,91[ab]	83,32 ± 10,91[b]
1,5	21	40,47 ± 8,01[b]	59,53 ± 8,01[b]
3	21	100 ± 0,00[c]	0,00 ± 0,00[a]
6	21	4,76 ± 4,76[a]	95,24 ± 4,75[c]

[a, b, c]Different superscripts within the same column indicate significant difference

In our experiment, we also observed the dead oocyte in each incubation period as indicated by the presence of red oocyte nucleus. The number of dead oocyte in incubation time of 0-2 h was recorded as the highest and significantly different from that of other incubation periods ($P<0.05$). The dead oocyte nucleus appeared red in color using propidium iodide (PI) staining since the nuclei staining dye PI could pass through a dead cell membrane and produce red color in oocyte nucleus (Murti, 2013).

The experimental results discussing the effects of sucrose concentrations on oocyte nucleus swelling is presented in Table 3. The results showed that the highest swelling percentage (100%) was attributed to the sucrose concentration of 3%, while the lowest percentage (4.75%) was found at sucrose level of 6%. Statistically, we also found that the swelling percentage attained at 3% of sucrose was significantly different in comparison with other sucrose levels ($P<0.05$). Accordingly, the addition of sucrose at 3% was considered as the optimum level to induce oocyte nucleus swelling, which visualize easily and applicable for enucleation procedures. The swollen nucleus oocyte is exhibited in Figure 1F. Wang *et al.* (2001a) reported that the swollen nucleus oocyte on mice was present in around chromosome at MII stage, thus the area near spindles are transparent and appear like "0".

In this study, the oocyte at GV stage was only found at incubation time of 0-2 h. The presence of oocyte at GV stage was considered low, which may be influenced by location and time of oocyte collection after superovulation. As reported by Mahmoudi *et al*. (2005), the oocyte from mice ovary was collected after 44 h of PMSG injection. Meanwhile, in our experiment, the oocyte was collected from oviduct after 48 h of PMSG injection and followed by 15-16 h of hCG injection.

Oocyte at MI stage can be observed in each incubation time. Fulka *et al.* (1995) reported that MI stage begins after 4 h of incubation in culture medium. Similar to MI, A/T I stage was also found in each incubation time. This is augmented by results reported by Fulka *et al.* (1995), finding that oocyte at A/T I could be seen after 9 h of incubation, indicated by formation of two nuclei. Furthermore, oocyte at MII stage could be observed after 8-10 and 12-14 h of incubation. Fulka *et al.* (1995) reported that MII oocyte on mice begins with the formation of polar body I (PB I) that appears after 11 h of incubation.

MII oocyte constitutes an indispensable component for SCNT techniques, since the technique is dependent on cytoplasmic condition in the mature and unfertilized oocyte (under inactive phase) (Mitalipov *et al.*, 2007). At MII stage, the nucleus is close to the first polar body (PB I). Nevertheless, Liu *et al*. (2002) found that only 31% of the oocyte indicated their nuclear components close to PB I. Consequently, PB I is used by most researchers to localize the nuclear material. This could decrease the enucleation rate. Even when 30% amount of cytoplasm is removed, the position of nucleus is not easy to detect. Therefore, a modified method by incorporation of sucrose in the medium is proposed in order to easily determine the position of nucleus in the cytoplasm. The hypertonic sucrose solution enables to shrinkage the oocyte and alters the concentration of cytoplasmic protein, leading to meaningful changes in membrane properties and cytoskeleton dynamics (Wang *et al.*, 2001a).

The use of optimum sucrose concentration for visualization of the oocyte nucleus in M2 culture (Wang *et al.*, 2001a) and CZB (Murti, 2013) media is found not different in comparison with the optimum level of sucrose (3%) in M199 medium. The sucrose-enriched medium could induce hypertonic condition, generating a projection as the result of interaction between actin filament in chromosome surface and spindle and/or attached chromosome, which is in turn, resulting in nuclear swelling (Murti, 2013). Wang *et al.* (2001a) reported that nuclear swelling in mouse oocyte is observed in around chromosome, which makes the area near spindle transparent like "∞" and "0" shape. However, the sucrose treatment may demonstrate dissimilar reactions on oocytes from other species. For instance, nuclear swelling of bovine oocyte was observed as peak-like shape (Liu *et al.*, 2002).

The materials comprise spindle fibers and cytoplasm in oocyte are different. This is the reason why sucrose treatment enables to promote alteration on the spindle instead of cytoplasm-composing components in oocyte nucleus; thus, the spindle is visible under inverted microscope. The increased number of nucleus swelling due to sucrose treatment of 0-3% was able to enlarge perivitellin space, which increased the oocyte viability during cultured in the medium. Sucrose treatment at 3% is considered non-toxic and does not promote cell damage, but at higher concentration (6%), the swelling of oocyte nucleus is reduced. This presumably indicates that the excessive sucrose level (hypertonic condition) can damage the oocyte membrane. Sucrose treatment has been widely applied in embryonic cryopreservation and nuclear transfer for livestocks. Additionally, it is also applicable to asssess the oocyte quality prior to micromanipulation (Wang *et al.*, 2001a).

CONCLUSION

The optimum incubation time for mouse oocyte to reach MII stage was found at 12-14 h, resulting in percentage of 57.14±12.67%. Meanwhile, the optimum condition for nuclear swelling was prepared at sucrose concentration of 3% with the swelling rate of 100±0.00%.

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