The Effects of Duration of the *In Vitro* Maturation Process on the Maturation Level and Apoptosis of Kacang Goat

M. Nursadida, W. Widjiati, I. Mustofa, E. Safitri, S. Susilowati and T. Wahyu Suprayogi*

Abstract

The success of *in vitro* fertilization and embryo culture depends on the success of *in vitro* maturation. However, standard culture conditions usually increase reactive oxygen species (ROS), which have been implicated as a major cause for reduced embryonic development. It is well-known that higher than physiological levels of ROS trigger granulosa cell apoptosis and thereby reduce the transfer of nutrients and survival factors to oocytes, leading to apoptosis. This study aimed to determine the optimal timing of oocyte maturation and its relationship to DNA fragmentation. Ovaries were collected from a slaughterhouse and the follicles aspirated. The cumulus oocyte complexes were divided into groups and transferred to a maturation medium, where they were maintained for 18 hours (P1), 22 hours (P2) and 24 hours (P3) to evaluated maturation rate. Matured oocytes were characterized as oocytes that reached the MII stage. Matured oocytes were counterstained with terminal deoxynucleotidyl transferase nick-end labelling (TUNEL). The results showed that the maturity rate of Kacang goat oocytes reached 46% after 18 hours, 77% after 22 hours, and 63% after 24 hours. However, the results showed that the expression of DNA fragmentation in P2 (2.4 ± 0.89) were significantly different from P1 (5.4 ± 2.61) and P3 (9.0 ± 2.12). In conclusion, the optimal timing of *in vitro* maturation of Kacang goat oocytes is 22 hours.

Key words: *IVM; DNA fragmentation; optimal timing; Kacang goat; food production*

Introduction

Biotechnology methods have been developed and used to improve the efficiency of livestock reproduction (Susilowati et al., 2018). *In vitro* production of embryos refers to the use of laboratory techniques to produce embryos (Asad et al., 2018). The *in vitro* embryo production (IVEP) method involves three basic steps: *in vitro* maturation (IVM) of primary oocytes collected from follicles, fertilization of matured secondary oocytes, and culture of potential probable

Milla NURSADIDA, Widjiati WIDJIATI*, (Corresponding author, e-mail: widjiati@fkh.unair.ac.id), Imam MUSTOFA, Erma SAFITRI, Suherni SUSILOWATI, Tri WAHYU SUPRAYOGI, Department of Veterinary Science Faculty of Veterinary Medicine Universitas Airlangga, Indonesia

embryos to the blastocyst stage (Avelar et al., 2012). The efficiency of IVEP is limited as it can generate about 30–50% of viable blastocysts (Fernandes-Franca et al., 2020). *In vitro* maturation (IVM) is the initial step of the IVEP method (Maskura et al., 2020). In the *in vitro* maturation process, a stable culture system and quality of oocytes are needed as a good source of oocytes.

There are several things that can increase the success of oocyte maturation, depending on the culture condition used, such as hormonal stimulation in the medium, medium supplementation, species characteristics, and time of exposition of maturation (Crocomo et al., 2020; Widjiati et al., 2020). Optimal culture timing is helpful for developmental competence of IVM oocytes (Yu et al., 2016). When culture time is too short, the maturation of the cytoplasm and the nucleus is not synchronized, which affects the subsequent development potential of the embryo (La et al., 2019). A long storage time can reduce the quality of oocytes (Widjiati et al., 2020). After 24 hours of IVM, ovine oocytes showed signs of degeneration, such as ooplasm shrinkage and granulation, and a disorganization in mitochondrial distribution (Maximo et al., 2012).

Mitochondria distribution is a process mediated by a network of microtubules, and requires a high ATP level supplied through mitochondrial metabolism (Ge et al., 2012). Mitochondria modulate the amplitude and kinetics of local and bulk cytoplasmic Ca^{2+} changes; in addition, they are dependent on Ca^{2+} signals for their own functionality, especially their capacity to produce ATP. mCa^{2+} has been found to be an important component controlling the rate of ATP production, and it controls the occurrence of MPT, which plays a role in mitochondrial control of apoptosis (Zhao et al., 2015). This study therefore aimed to determine the optimum time of oocyte *in vitro* maturation and its correlations with apoptosis.

Materials and methods

Ethics Approval

This research received ethical approval with permit number 1.KEH.35.03.2021 by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Universitas Airlangga.

Materials

The study materials included the following: Saline solution (0.9%) Otsu®. The maturation medium used Minimum Essential Medium (MEM, GibcoBRL®), HEPES (Sigma-Aldrich®) stored at 4-5°C with 7.2 pH, Bovine Serum Albumin (BSA), Sodium bicarbonate (Sigma-Aldrich®, Steinheim, Germany), Kanamycin sulfate (Bioplus), Pregnant Mare Serum Gonadotropin (PMSG) (PG600®, Canada), Hormone Chorionic Gonadotropin (hCG) (PG600®, Canada). Hyaluronidase (HYASE-10x™, Vitrolife®) for denudation, and apoptosis expression examination with immunocytochemistry (TACS-XL® DAB In Situ Apoptosis Detection Kit).

Collection and processing of ovaries

The ovaries were transported from slaughterhouses to the laboratory using a thermos. They were placed into a glass beaker containing 0.9% saline solution (0.9%) with penicillin G (75 µg/mL) and streptomycin sulfate (50 µg/mL) at 37°C. After trimming the surrounding tissues, ovaries were washed again with saline solution.

Collection of cumulus oocyte complexes

Cumulus oocyte complexes (COC) were aspirated in the follicles with a

diameter of 2–6 mm using a 10 mL disposable syringe with an 18-G needle containing Phosphate Buffered Saline (PBS) medium. All aspirations were put into a Petri dish for evaluation under 40× magnification on an inverted microscope. After evaluation, COCs containing healthy oocytes were selected based on their morphological appearance of a uniformly granulated cytoplasm surrounded by a multilayer of compact cumulus cells least three layers thick, and washed with PBS 3 times.

In vitro maturation of COCs

The maturation medium was prepared with Minimum Essential Medium (MEM) supplemented with 3% bovine serum albumin (BSA), 0.15 IU/mL Pregnant Mare Serum Gonadotropin (PMSG) and 15 IU/mL human chorionic gonadotropin (hCG). COCs were placed in 50 µL maturation droplets of maturation medium under paraffin oil, drops were made on a 35 mm disposable Petri dish (each drop contained ±3–4 oocytes). They were cultured in an incubator with environmental conditions of 5% CO_2, temperature of 37.5°C, and maximum humidity level (95–99%) for 18 hours, 22 hours or 24 hours.

Oocyte maturation of Kacang goat

After 18 h, 22 h or 24 h of culture, observations were made to see the level of oocyte maturity using a Nikon Diaphot 300 microscope at 100x and 400x magnification. To observe Polar body I, a denudation was performed to remove cumulus cells. Chemical denudation usually uses the enzyme hyaluronidase (HYASE-10x™, Vitrolife®), and the oocyte is immersed in a medium containing the hyaluronidase enzyme and left for 30 seconds then transferred to the culture medium. The maturity level of oocytes is measured by the number of oocytes that have undergone first polar body (PBI) release as a benchmark for oocyte maturation divided by the total number of oocytes multiplied by 100%.

Examination of apoptosis using immunocytochemistry

Oocytes were placed on a glass object coated with poly-L-lysine and then covered with a cover glass. Fixation of oocytes was conducted by putting the glass object into a container with 100% acetic acid (glacial) and absolute ethanol at a ratio of 1:3 for a minimum of 24 h before immunocytochemical colouring was performed. Slides were then deparaffinized in xylol 1, xylol 2, ethanol absolute 1, ethanol absolute 2, 90% ethanol, 80% ethanol, 70% ethanol, 30% ethanol, each for 5 minutes, then slides were washed in distilled water before staining with PBS for 5 minutes. Slides were covered with 50 µL proteinase K for 15–30 minutes or with 50 µL cytonin for 30 minutes. Then slides were washed with deionized water every 2 minutes before being dropped into quenching solution for 5 minutes and then washed with PBS for 1 minute. Slides were stained with 1xTDT labelling buffer for 5 minutes and then dropped into 50 µL Labelling reaction mix. This was followed by incubation at 37°C in a damp place for 60 minutes. The sample was dripped with 1xTDT stop buffer for 5 minutes and then washed with sterile distilled water. After covering the slides with SA-HRP solution, they were incubated at 37°C in a damp place for 10 minutes. This was followed by rinsing 2x with PBS for 2 minutes before dropping into DAB Solution for 2–7 minutes, and then rinsing 2x with sterile distilled water for 2 minutes. Slides were dropped into methyl green for 30 seconds to 5 minutes, and then soaked in a mixture 2x distilled water, 95% ethanol

and absolute ethanol. Finally, slides were mounted using entellan.

Observation of apoptosis expression

Apoptosis expressions were observed using an CX41 microscope (Olympus, Japan). The results of each expression were assessed semi-quantitatively according to the modified Remmele method (Nowak et al., 2007). The index Remmele scale was the result of multiplying the percentage score of immunoreactive cells with the colour intensity scores produced on the cell, as shown in Table1.

Statistical analysis

All data of oocyte maturity level and apoptosis were subjected to one-way ANOVA followed by Duncan test (IBM SPSS Statistics 25), and differences at $P<0.05$ were considered statistically significant.

Results

Oocyte maturity level

The cumulus oocytes complexes that were successfully collected amounted to 25 oocytes for each group meeting the requirement for *in vitro* maturation in a different time period. The level of oocyte maturity obtained from maturation differed among treatment groups 1, 2 and 3. The number of mature oocytes marked by the formation of Polar Body I in treatment group 2 was higher than both groups 1 and 3 (Table 2). According to Table 2, oocytes were able to experience *in vitro* maturation at 22 hours with a percentage of 77%. The mature oocytes are marked by the formation of Polar body I.

Apoptosis examination using immunocytochemistry

Apoptosis of oocytes in Kacang goat following *in vitro* maturation was expressed by brown colouration on both structures that indicated the bond of antigen and antibody as visualized by chromogenic change. According to Table 2, group 1 (T1) differed significantly from T2 ($P<0.05$), which also differed significantly from T3 ($P<0.05$). The expression of apoptosis in T2 (2.4 ± 0.89) was the lowest compared to the groups T1 (5.4 ± 2.61) and T3 (9.0 ± 2.21).

Discussion

The result showed that the highest level of oocyte maturity was found in treatment group 2, after *in vitro* maturation of 22 hours (mean 77.14). This result was significant different ($P<0.05$) from treatment group 1 (45.71%), though group 3 (62.86%) did not significantly differ from groups 1 or 2. The release of first polar body (PBI) has been regarded as the benchmark of oocyte maturation. However, our results indicate that spindle

Table 1. The index Remmele scale

(a)		(b)	
Score 0	No positive cells	Score 0	No colour reaction
Score 1	Positive cells less than 10%	Score 1	Low colour intensity
Score 2	Positive cells between 11% and 50%	Score 2	Medium colour intensity
Score 3	Positive cells between 51% and 80%	Score 3	Strong colour intensity
Score 4	Positive cells more than 80%		

Table 2. Mean and Standard Deviation Maturation Level and Apoptosis of Kacang Goats

Group	Maturation Level (%) (Mean ± SD)	Apoptosis (Mean ± SD)
T1	45.71 ± 15.12^{a}	5.4 ± 2.61^{a}
T2	77.14 ± 17.99^{b}	2.4 ± 0.89^{b}
T3	62.86 ± 13.80^{ab}	9.0 ± 2.12^{c}

Different superscripts in the same column show significant differences ($P<0.05$), T1: IVM 18 hours, T2: IVM 22 hours, T3: IVM 24 hours

assembly may be very different in these seemingly matured oocytes and is closely related to the timing of *in vitro* maturation (Yu et al., 2011). Adequate extension of IVM time can promote the necessary process of oocyte maturation, increase the rate of nuclear maturation in immature oocytes, and significantly improve *in vitro* the developmental potential of oocytes and the rate of high-quality embryos (La et al., 2019). Souza-Fabjan et al. (2014) reported that 22 hours is the optimal time of *in vitro*

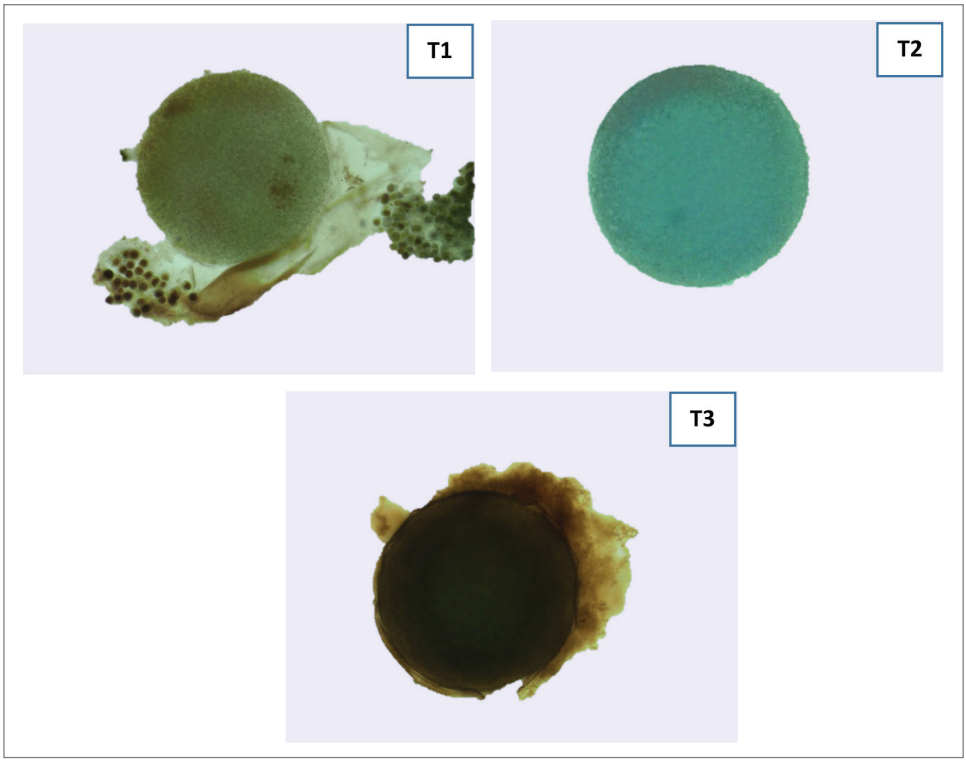

Figure 1. Expression of apoptosis followed by in vitro maturation in different times, 18 h (T1), 22 h (T2), 24 h (T3). (T1) Apoptotic expression was weak in oocytes, (T2) apoptosis was not seen in oocytes so that the colour counter staining methyl green was green, (T3) apoptosis was very strong in oocytes as expressed in the strong brown colour due to antigen, antibody and chromogenic binding (Nikon Microscope type Ellipse Ci Camera at 100x magnification).

maturation. On other hand, Maximo et al. (2012) reported that 18 h was sufficient for the oocyte to complete maturation. They also suggested that degeneration signs may be due to the excessive time in IVM (24 h), which may disrupt the oocyte structures to induce apoptosis.

In addition to maturation data, oocytes were examined for apoptosis after *in vitro* maturation. The results on apoptosis are presented in Table 2 and Figure 1. Brown colouration indicates an oocyte that experienced apoptosis, which would influence the nutritional supply from the cumulus to the oocyte. This led to the inability for the oocyte to achieve perfect maturity. Whereas, oocytes that did not experience apoptosis were expressed by staining counter colour that is methyl green. The oocytes did not experience apoptosis, enabling the oocyte to achieve the optimum time maturity in the metaphase II stage.

Based on the research, the lowest expression of apoptosis in treatment group T2 was significantly different from groups T1 and T3. Many factors influenced apoptosis to take place and accelerate the process. Excessive culture time leads to the aging of oocytes and increased level of associated genetic risks (La et al., 2019). A recent study suggested that a major cause for impaired developmental competence of aging oocytes is the induction of oxidative stress, which triggers many cascades that influence oocyte quality, such as apoptosis (Zhang et al., 2019). Oxidative stress could be due to cytochrome binding to apoptotic protease-activating factor-1 (APAF-1) released from the mitochondria; then procaspase-9 can activate caspase-9.43. Caspase-9, which serves as an apoptosis initiator, is dimerized, triggering feedback by inhibiting BCL-2 release and then binding to procaspase-3 to activate caspase-3 (Safitri et al., 2023). Caspase3, which acts as an executor, may facilitate endonuclease activation and cytoplasmic proteases that may lead to nuclear DNA fragmentation and degradation of cytosol protein. The final result in the fragmentation process is the formation of apoptotic bodies containing intracellular organelles, and the expression of phosphatidylserine that trigger phagocytosis (Samik and Safitri, 2017).

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References

1. ASAD, L., M.A.M.Y. KHANDOKER, S.A.M. HOQUE and S. SAHA (2018): Effect of follicular fluid on in vitro maturation, fertilization and development of goat embryos using fresh semen. Int. J. Agric. Innov. Res. 6, 1473-2319.
2. AVELAR, S. R. G., R. R. MOURA, F. C. SOUSA et al. (2012): Oocyte Production and In Vitro Maturation in Canindé Goats Following Hormonal Ovarian Stimulation. J. Anim. Reprod. 9, 27-32.
3. CROCOMO, L. F., F. ARIU, L. BOGLIOLO, D. BEBBERE, S. LEDDA and S. D. BICUDO (2020): Roscovitine Use For The Delay of Meiotic Progression In prepubertal sheep oocytes. Pesqui. Agropecu. Bras. 55, 1-8. 10.1590/S1678-3921.pab2020.v55.01585
4. FERNANDES-FRANÇA, P. H., M. V. OLIVEIRA-SANTOS, G. P OLIVEIRA-LIRA, A. AZEVEDO-BORGES and A. FERNANDES-PEREIRA (2019): Influence of different protein supplements on the recovery and in vitro maturation of bovine oocytes. Rev. Colomb. Cienc. Pecu. 33, 172-181.
5. GE, H., T. L. TOLLNER, Z. HU, M. DAI, X. LI, H. GUAN, D. SHAN, X. ZHANG, J. LI, C. HUANG and Q. DONG (2012): The importance of mitochondrial metabolic activity and mitochondrial dna replication during oocyte maturation in vitro on oocyte quality and subsequent embryo developmental competence. Mol. Reprod. Dev. 79, 392-401. 10.1002/mrd.22042.
6. LA, X., J. ZHAO and Z WANG (2019): Clinical Application of In Vitro Maturation of Oocytes. Embryology - Theory and Practice page 3. 10.5772/intechopen.87773.

7. MAKSURA, H., N. AKON, N. ISLAM, I. AKTER, A. K. MODAK, A. KHATUN, H. ALAM, A. HASHEM, R. AMIN and M. MONIRUZZAMAN (2021): Effects of estradiol on in vitro maturation of buffalo and goat oocytes. Reprod. Med. Biol. 20, 62-70. 10.1002/rmb2.12350
8. MÁXIMO, D. M., I. G. MARTINS SILVA, R. G. MONDADORI, J. P. NEVES and C. M. LUCCI (2012): Ultrastructural characteristics of sheep maturation oocytes during in vitro (IVM). Small Rumin. Res. 105, 210-215. 10.1016/j.smallrumres.2011.11.021
9. NOWAK, M., J. A. MADEJ and P. DZIEGEIL (2007): Intemsity of Cox 2 expression in cell of soft tissue fibrosarcomas in dog as related to grade of tumor malignation. Bull. Vet. Inst. Pulawy 51, 275-279.
10. SAFITRI, E., H. PURNOBASUKI, M. T. E. PURNAMA and S. CHHETR (2023): Role of apoptotic inhibitors, viability, and differentiation in low oxygen tension of mesenchymal stem cells cultured in a rat model of ovarian failure. F1000Research. 12, 1-15. 10.12688/f1000research.124919.1
11. SAMIK, A. and E. SAFITRI (2017): Potency of mycotoxin binders on MDA level, expressions of caspase 9 and caspase 3 in the uterus of mice exposed to zearalenone. Iraqi J. Vet. Sci. 31, 29-33. 10.33899/ijvs.2017.126707
12. SOUZA-FABJAN, J. M. G., Y. LOCATELLI, N. DUFFARD, E. CORBIN, J.-L. TOUZÉ, C. E PERREAU, J. F. BECKERS, V. J. F. FREITAS and P. MERMILLOD (2014): In vitro embryo production in goats: slaughterhouse and laparoscopic ovum pick up–derived oocytes have different kinetics and requirements regarding maturation media. Theriogenology 81, 1021-1031. 10.1016/j.theriogenology.2014.01.023.
13. SUSILOWATI, S., T. W. SUPRAYOGI, RIMAYANTI, T. HERNAWATI and SUHARSONO (2018): The addition of L-Arginine in capacitation media to motility, viability, and spermatozoa capacity of goats. Int. J. Chem. Tech. Res. 11, 13-18.
14. WIDJIATI, Z. F., N. DARSINI, V. F. HENDRAWAN, E. M. LUQMAN and S. B. SUMITRO (2020): The Role of Cumulus in the in vitro Maturation Process towards the Maturation Level of Kacang Goats (*Capra aegagrushircus*). J. Int. Dent. Med. Res. 13, 1211-1216
15. YU, Y., J. YAN, Z. C. LIU, L. Y. YAN, M. LI, Q. ZHOU and J. QIAO (2011): Optimal timing of oocyte maturation and its relationship with the spindle assembly and developmental competence of in vitro matured human oocytes. Fertil. Steril. 96, 73-78. 10.1016/j.fertnstert.2011.04.077
16. ZHAO, XUE-MING, H.-S. HAO, W.-H. DU, S.-J. ZHAO, H.-Y. WANG, N. WANG, D. WANG, Y. LIU, T. QIN and H.-B. ZHU (2015): Melatonin inhibits apoptosis and improves the developmental potential of vitrified bovine oocytes. J. Pineal Res. 60, 132-141. 10.1111/jpi.12290.
17. ZHANG, B., X. MIANQUN, X. S. YANG, Y. ZHANG, Y. MIAO, Y. CHEN and Z. CUI (2019): Coenzyme Q10 ameliorates the quality of postovulatory aged oocytes by suppressing DNA damage and apoptosis. Free Radic. Biol. Med. 143, 84-94. 10.1016/j.freeradbiomed.2019.08.002

Učinci trajanja postupka *in vitro* sazrijevanja na razinu sazrijevanja i apoptozu u kacang koza

Milla NURSADIDA, Widjiati WIDJIATI, Imam MUSTOFA, Erma SAFITRI, Suherni SUSILOWATI, Tri WAHYU SUPRAYOGI, Department of Veterinary Science Faculty of Veterinary Medicine Universitas Airlangga, Indonesia

Uspjeh *in vitro* oplodnje i kulture embrija ovisi o uspjehu *in vitro* sazrijevanja. Međutim, standard uvjeta kulture obično povećava reaktivne kisikove spojeve (ROS), za što se sumnja da je jedan od glavnih uzroka smanjenog razvoja embrija. Dobro je poznato da razine ROS veće od fizioloških razina izazivaju apoptozu granuloznih stanica i time smanjuju prijenos nutrijenata i faktora preživljavanja na oocite, što dovodi do apoptoze. Ovo istraživanje imalo je za cilj otkriti optimalno vrijeme sazrijevanja oocita i njegov odnos s DNK fragmentacijom. Prikupljeni su jajnici iz klaonice i aspirirani su folikuli. Kompleksi oocita-cumulus (COCs) podijeljeni su u skupine i preneseni u medij za sazrijevanje gdje su držani 18 sati (P1), 22 sata (P2) i 24 sata (P3) za procjenu postotka sazrijevanja. Zreli oociti su okarakterizirani putem oocita koji su dosegnuli MII fazu. Zreli oociti obojani su TUNEL metodom. Rezultati su pokazali da je postotak zrelosti koju su dosegli oociti kacang koze nakon 18 sati bio 46 %, nakon 22 h 77 % i nakon 24 h 63 %. Međutim, rezultati su pokazali da se ekspresija DNK fragmentacije u P2 (2,4 ± 0,89) značajno razlikovala od P1 (5,4 ± 2,61) i P3 (9,0 ± 2,12). Zaključno, optimalno vrijeme *in vitro* sazrijevanja oocita kacang koze je 22 sata.

Ključne riječi: *in vitro sazrijevanje (IVM), DNK fragmentacija, optimalno vrijeme, kacang koza, proizvodnja hrane*