J I T A A
Journal of the Indonesian Tropical Animal Agriculture
Accredited by Ditjen Riset, Teknologi dan Pengabdian kepada Masyarakat No. 164/E/KPT/2021

J. Indonesian Trop. Anim. Agric.
pISSN 2087-8273 eISSN 2460-6278
http://ejournal.undip.ac.id/index.php/jitaa
47(3):235-243, September 2022
DOI: 10.14710/jitaa.47.3.235-243

Maturation rate of sheep oocytes cultured in suboptimal environment using CO_2 exhaled by human and glutathione addition

S. Prastowo[1,2] * and R. Widyastuti[3]
[1]*Animal Science Department, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Indonesia.*
[2]*Tropical Animal Breeding, Health, and Reproduction Research Group, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Indonesia.*
[3]*Laboratory of Animal Reproduction and Artificial Insemination, Department of Animal Production, Animal Husbandry Faculty, Universitas Padjadjaran, West Java, Indonesia*
**Corresponding e-mail: prastowo@staff.uns.ac.id*

Received September 24, 2021; Accepted March 10, 2022

ABSTRACT

This study aimed to identify the effect of the use of CO_2 exhaled by human (CEbH) in in vitro maturation (IVM) with the addition of Glutathione also known as GSH on the oocytes maturation rate. It is known that CO_2 derived from human respiration can be used as alternative support for IVM of oocytes. However, this method is categorized as using a suboptimal culture environment, and it results in increased production of reactive oxidative species (ROS). Thus, the addition of the antioxidant GSH is needed. In total, 273 oocytes were cultured in three groups: 5% CO_2 (control) and CEbH with 0 mM and 1 mM of GSH addition, respectively. The results showed that the control group produced a better maturation rate ($p < 0.05$) compared to the remaining two groups (65.92% vs. 24.44% and 30.61%), which used CEbH. Statistically, 0 mM GSH vs. 1 mM GSH did not show any significant differences ($p > 0.05$). However, 1 mM GSH led to more mature oocytes (24.44% vs 30.61%) compared to the group without GSH. In conclusion, IVM using CEbH supports oocyte maturation, and the addition of GSH can improve the maturation rate in a suboptimal culture environment.

Keywords: Suboptimal culture environment, IVM, CO_2 exhaled by human, Glutathione, Sheep oocytes

INTRODUCTION

In vitro embryo production (IVP) is a reproductive technology that can be used to improve livestock genetic quality. This IVP system enables in vitro fertilization for embryo production and is performed in the culture system, which mimics the female reproductive tract environment. The success of assisted reproduction technology implementation—and embryo production in particular—generally depends on the optimal development of pre-implantation embryos in cul-

ture system (Itoi *et al.*, 2012; Vajta *et al.*, 2010; Wrenzycki, 2016). As part of the in vitro culture (IVC) system, the media and the incubator each play a role in determining the quality of the resulting embryo (Wu *et al.*, 2017). Hence, the system is dependent on the microenvironment and uses CO_2 incubators to grow the oocytes or embryos in the culture medium. In this stage, IVC is commonly performed in 5%–6% CO_2, at 37–38 °C, and under 95% humidity to maintain the pH (Amin *et al.*, 2014). However, this would become a considerable obstacle for culture work in the case of limited or no CO_2 supply.

The previous study demonstrated that CO_2 exhaled by human (CEbH) could be applied in an IVC system in order to support porcine oocyte maturation as well as its embryo development (Cao *et al.*, 2018). The study demonstrated the possibility of using an alternative CO_2 source for IVM, although a specific concentration of CO_2 cannot be controlled and measured. Earlier reports (Raczek and Adamczyk, 2004; Vajta *et al.*, 1997) have shown that human-exhaled air has low CO_2 concentration (<5%) and suggested triggering the reactive oxygen species (ROS) in IVC system. The increased production of ROS could hamper oocyte development competence as a result of oxidative stress (OS) (Du Plessis *et al.*, 2008).

To protect themselves against ROS, cells have a defense mechanism system that entails the production of an antioxidant (Amin *et al.*, 2014). However, the imbalance between the antioxidant and ROS production could potentially change the OS balance mechanism, thus impacting the mitochondrial activity. Low mitochondrial activity has been found to affect bovine embryo metabolism and its development competence (Amin *et al.*, 2014; Prastowo *et al.*, 2016). To overcome the OS, an antioxidant - such as Glutathione (GSH) - could be added to the culture system (Ali *et al.*, 2003; Truong and Gardner, 2017), which enables the scavenging of ROS and protects cells from OS. GSH is normally secreted by cumulus cells (CC) during oocyte development and deposited in oocyte cytoplasm, and it has a high association with oocyte development capacity (García-Martínez *et al.*, 2020; Jiao *et al.*, 2013).

The aforementioned studies have shown the possibility of using CEbH as an alternative source to support IVC system including IVM. Due to its low CO_2 concentration, the culture using CEbH as a source would lead to OS. As such, the addition of GSH as an antioxidant in the culture system would benefit and support oocyte development. This study explores the possibility of whether using CEbH combined with GSH addition would result in better IVM outcome. To do so, Indonesian local sheep oocytes are used as the study model. The study results have the potential to be used as an alternative method for IVM when a limited availability of CO_2 sources is present.

MATERIALS AND METHODS

Collection of Cumulus-oocyte Complexes

Sheep ovaries were collected from a local slaughterhouse and transported to the laboratory using physiological saline (NaCl 0.9%) supplemented with 100 IU/mL penicillin (Meiji, Japan) and 0.1 mg/mL streptomycin (Meiji, Japan) at 30 –37 °C within 4 hours. Cumulus-oocyte complexes (COCs) were then collected using the slicing method in Dulbecco's Phosphate-Buffered Saline (DPBS) (Sigma, USA) and supplemented with 5% fetal bovine serum (FBS) (Gibco, USA), 100 IU/mL penicillin (Meiji, Japan), and 0.1 streptomycin mg/mL (Meiji, Japan). According to the previous methods (Widyastuti *et al.*, 2017; Widyastuti and Rasad, 2015), the COCs selected for culture were classified based upon the CC layer, and only oocytes with homogenous cytoplasm were selected. In this study, COCs with a minimum of two CC layers were selected for the IVM culture program with different culture environment systems.

In vitro maturation and culture environment treatment

In total, 273 COCs were selected and briefly transferred to a 35 mm dish (Nunclon®; Sig-

ma USA). This was then followed by two washes using maturation medium containing tissue culture medium 199 (TCM 199; Sigma USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 IU/mL penicillin (Gibco, USA), 0.1 mg/mL streptomycin (Gibco, USA), 10 mg/mL human chorionic gonadotrophin (hCG) (Chorulon, International Intervet BV Boxmeer Holland), and 0.01 mg/mL follicle stimulating hormone (FSH) (Sigma, USA).

The earlier study (Cao *et al*., 2018) shows the capability of CO_2 derived from human exhaled air to support porcine oocytes maturation in vitro by using sealed sterile aluminum bags and culture disc. In brief, CO_2 was blown into sterile aluminum bags then connected to the oocyte culture disc using a pipe. In our study, the CO_2 from human exhaled air was prepared by blowing directly into a falcon tube, already filled with oocytes and maturation medium, using a sterile pipe connected to a filter. Falcon tubes were then sealed and put in the incubator for culture at 38 °C for 22–24 hours. In this study, we performed three culture environments (groups): the optimal culture condition using 5% CO_2 (control), and two types of suboptimal culture conditions that were CEbH with a 0 mM and 1 mM l-glutathione (GSH; Sigma, USA) addition, respectively.

All of the oocytes were cultured in 5 mL falcon tubes, and each tube contained 5–10 COCs in the respective treatments. Subsequently, the maturation rate was evaluated according to the number of extrusions of the first polar body in proportion to the number of matured oocytes (Widyastuti *et al.*, 2017).

Data analysis

The maturation rate data of the oocytes between the three groups were statistically analyzed using the chi-square (X^2) test. This, due to oocyte maturation data is categorical variable (mature vs undeveloped) and the data type is nominal. At this point, considering the goodness fit of statistical analysis, the most appropriate test is chi-square. The significant difference was decided at $p < 0.05$, and the analysis was performed with the help of the R statistical program (R Core Team, 2019).

RESULTS AND DISCUSSION

In total, 273 oocytes were collected from local sheep ovaries. Of the total, 144 oocytes were categorized as grade A, which was indicated by a total of more than three layers of CC (Figure 1A). A total of 129 oocytes were categorized as grade B (Figure 1B), which has the minimum of two CC layers. In the current study, we grouped grade A and B for IVM. After 22–24 hours, we are able to find mature oocytes in all of the culture environments, showing the capability of the oocytes to extrude their first polar body (Figure 2A). Meanwhile, we also found oocytes that were not able to develop into mature oocytes, as shown in Figure 2B.

Following post-IVM evaluation, this study shows that different culture environments give different maturation results, as tabulated in Table 1. Optimal culture conditions using 5% CO_2 resulted in a significantly better maturation rate ($p < 0.05$) as well as the production of less undeveloped oocytes when compared to the other two suboptimal culture conditions. In the culture group using CHeB - with nor without GSH addition - showed maturation rate and undeveloped oocytes post-IVM were statistically not significantly different ($p > 0.05$).

Table 1. The maturation rate of oocytes cultured with different environments

IVM culture condition	Number of oocytes (n)	Mature	Undeveloped
		---------- n (%) ----------	
5% CO_2	179	118 (65.92)[a]	61 (34.08)[b]
Exhaled CO_2 + GSH 0 mM	45	11 (24.44)[b]	34 (75.56)[a]
Exhaled CO_2 + GSH 1 mM	49	15 (30.61)[b]	34 (69.39)[a]
	χ^2	67.28	40.90

[a, b]values with different superscript letters in a column show significant difference ($p < 0.05$)

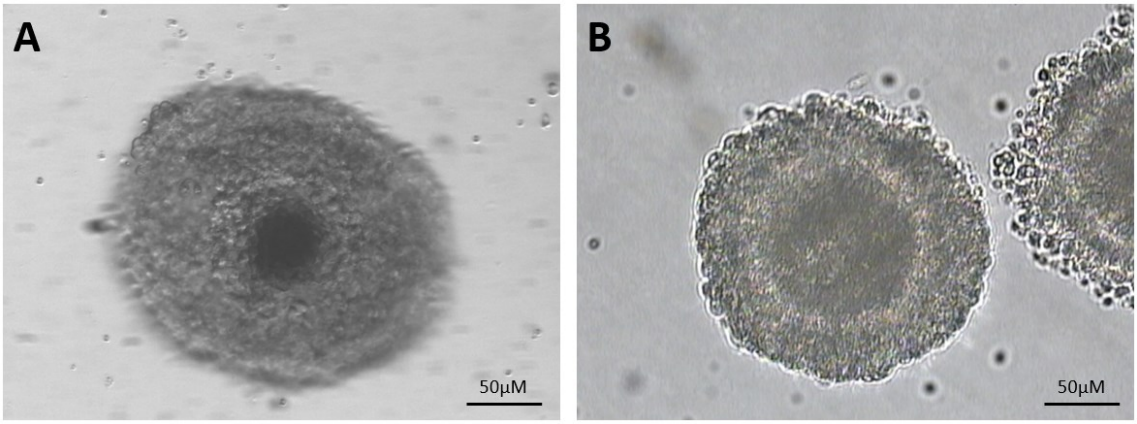

Figure 1. The quality of cumulus-oocyte complexes. (A = more than three cumulus cell layers, B = two cumulus cell layers)

The result in this study is in agreement with the previous study (Cao *et al.*, 2018), which demonstrated the ability of CO_2 from human-exhaled air to produce mature oocytes in vitro. Moreover, the addition of GSH was proven to have a beneficial effect on the culture environment by improving the number of matured oocytes. This result from our study is in line with the association of GSH with the oocyte maturation development competence reported in earlier studies (Yoshida, 1993; Yoshida *et al.*, 1993). Considering that the IVC environment using low CO_2 concentration would produce more ROS in the culture medium, the addition of GSH effectively enabled the counteracting and scavenging of the ROS produced in the culture medium. Increased ROS would impact the development kinetic of the embryos (Amin *et al.*, 2014) as well as their metabolism, indicated by low mitochondrial activity (Prastowo *et al.*, 2016). High ROS in the oocytes would decrease the maturation promoting factor (MPF), which ultimately leads to a decrease of oocyte competence to resume its meiotic process. As is generally known, the MPF functions to control mitosis and meiotic processes, and it also serves to induce the metaphase process in the oocyte (Tiwari and Chaube, 2017). As such, the presence of GSH during maturation acts as a gamete defense mechanism against ROS. Intracellularly high ROS levels cause damage to microtubules formation, which interrupts the oocyte meiotic process and the first polar body extrusion (Kala *et al.*, 2017).

In this study, we purposefully selected COCs that had more CC layers (Figure 1A and 1B), and the aim was to support better development during IVM. As reported in the previous study (Dadashpour Davachi *et al.*, 2012), the CC is a critical factor during oocyte maturation, as it plays a role in intercellular communication through the gap junction between the CC as well as between the CC and the oocyte (Appeltant *et al.*, 2015; Shimada, 2009). Next, it is explained that the calpain-calpastatin system in CC gene expression is responsible for signaling the pathway and metabolism transfer between the CC and the oocyte (Zhou *et al.*, 2016). This provides an explanation for the low oocyte grade, resulting in a low CC expansion rate and low extrusion of the first polar body. Less CC layers result in low oocyte metabolism support, thus causing low expansion and maturation rates of oocytes. Notably, CC is vital to producing the many metabolites needed for oocyte metabolism during development (Uhde *et al.*, 2018).

Compared to the IVM outcome using the CO_2 incubator (5% CO_2), our maturation result from the suboptimal culture condition is relatively lower (Table 1). This indicates a suboptimal in vitro incubation system when using CHeB. As mentioned before, this suggests that low CO_2 concentration may lead to increased ROS pro-

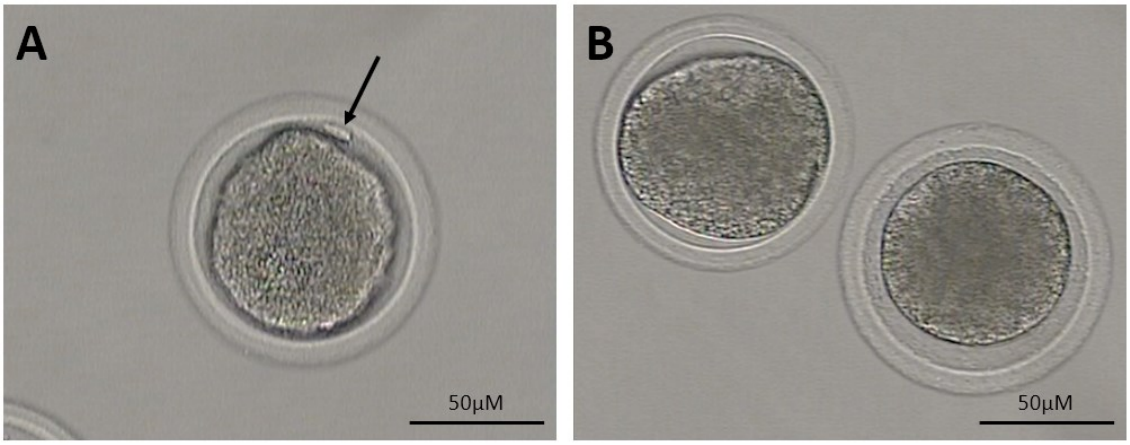

Figure 2. Oocyte status post in vitro maturation. A: Mature (arrow: first polar body); B: Undeveloped

duction. Higher ROS has been seen to reduce oocyte development competence and disturb the meiotic process (Du Plessis *et al.*, 2008). In this situation, an antioxidant is needed for OS protection during IVM. One of the more well-known antioxidants is GSH, and it is important for cell growth (Shi *et al.*, 2000).

In an in vivo condition, GSH is produced by oocyte follicular fluid (Gordon, 2003). On the contrary, in the in vitro system, GSH is only synthetized by cumulus cells (de Matos *et al.*, 1997). These differences lead to the lower intracellular GSH concentration in the in vitro condition compared to the in vivo condition. As seen in the present study, low intracellular GSH concentration is the cause of low oocyte maturation rates (Furnus *et al.*, 1998). That said, the addition of GSH in the current study (Table 1) proved to be capable of improving oocyte maturation rates.

GSH belongs to the category of non-enzymatic antioxidants. It is one of the naturally synthesized antioxidants that protect cells from ROS toxicity and regulate the intracellular redox balance (You *et al.*, 2010). It was reported that increasing GSH synthesis by adding a co-factor using thiol compounds in the IVM system can improve the maturation of oocytes (Nakamura *et al.*, 2011) and embryo development (Sun *et al.*, 2015). This is due to the fact that GSH plays an important role in DNA and protein synthesis as well as cell proliferation, and it is considered as the marker of oocyte quality (Torres-Osorio *et al.*, 2019). GSH is also known to have many important functions in intracellular physiology and metabolism. The most important role of GSH is to maintain the redox state in cells (He *et al.*, 2017; Xiong *et al.*, 2011), which protects them against harmful effects caused by oxidative injury (Lushchak, 2012). The protective effect of GSH against ROS is facilitated by the interaction with its associated enzymes, such as GPX and GSH reductase (He *et al.*, 2017).

In order to scavenge ROS, GSH maintains cellular oxidants and antioxidants balance via a redox state reaction (Schafer and Buettner, 2001). At this point, eliminating ROS requires GSH and NADPH. In brief, the reaction was initiated by conversion of H_2O_2 into H_2O through reduction reaction with the presence of GSH and catalytic enzyme namely glutathione peroxidase (GPX) resulting in oxidized glutathione (GSSG) (Trachootham *et al*., 2008). GSSG then efficiently reduced back to GSH with the help of NADPH resulting $NADP^+$. Later, *Glucose-6-phosphate dehydrogenase (G6PD) donate is* H^+ *to convert back the* $NADP^+$ *to NADPH and the cycle is continuously occurring. According to Jones (2002), the* cellular balance of GSH and GSSG provides a dynamic indicator of oxidative stress. Although GSH is synthesized continuously, these rates are generally slow relative to the turnover by the oxidation-

reduction cycle. During acute OS, GSH concentration decreases, and the associated increase in GSSG concentration results in an increased turnover of the GSH/GSSG cycle.

Oocytes are very sensitive to OS which has a detrimental effect on developmental arrest before zygotic genome activation and further apoptosis in the later stage. During oocytes maturation, mitochondria provide ATP for glutathione (GSH) production and also participate in the regeneration of NADPH and GSH during early development (Dumollard *et al*., 2009). Considering that function, then it is a logical reason to add GSH in oocyte or embryo culture medium aiming more number of development outcomes. External GSH is likely to add GSH internal concentration, providing the balance of GSH and GSSG then redox cycle can be maintained or could be restored.

As previously reported, the IVM system results in increased ROS; this, in turn, results in the triggering of excessive levels of cell apoptosis, the reduction of nutrient transfer, and the survival factor of cultured oocytes (Khazaei and Aghaz, 2017). In the natural system, ROS are neutralized by the defense mechanism of enzymatic and non-enzymatic antioxidants. That said, the balance between the ROS level and the antioxidant within IVM media is important for oocyte development. When excess ROS are present beyond physiological level, this may lead to OS and result in the deterioration of the quality of the oocytes (Chaube *et al.*, 2014). An increased level of ROS has been reported as associated with the cell cycle arrest in oocytes and embryos (Tripathi *et al.*, 2009). In order to manage ROS production, antioxidants can be added into the culture system with the aim of protecting cells from OS; this ultimately leads to an overall improvement in the culture outcome. Use of antioxidant molecules is thus vital to protect cells from high ROS levels and their negative effects (Torres-Osorio *et al.*, 2019). Selecting the best type and dosage of specific antioxidants is vital to the oocyte quality in IVM. Thus, it can be expected that the further process of embryo production will result in a good outcome as well.

The results of the current study present insight on the use of CHeB as an alternative in an IVC environment. However, it must be taken into consideration that the oocytes development result is not comparable to the optimal culture environment using 5% CO_2. The addition of the antioxidant potentially improves the culture result. However, its optimal concentration in the culture medium must be optimized in further studies.

CONCLUSION

Based on the results, it can be concluded that IVM using CO_2 exhaled by human supports oocytes in reaching the maturation stage. However, this culture environment is categorized as a suboptimal culture condition. The addition of GSH in this study improved the maturation rate of oocytes cultured in the suboptimal condition, but this improvement was still lower when compared to the optimal culture environment using 5% CO_2.

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