COMPARATIVE EFFECTS OF CUMULUS CELL CO-CULTURE AND CONDITIONED MEDIUM ON IN VITRO MATURATION OF MURINE OOCYTES

NIK ABD RAHMAN FAHMI, L.[1], REDZUAN NUL HAKIM, A.R.[1,2*], MUHAMMAD LOKMAN, M.I.[1], AND MUHAMMAD ALIF, M.[1]

1 Institute of Planetary Survival for Sustainable Well-being, International Islamic University Malaysia, 25100 Kuantan, Pahang, Malaysia

2 Kulliyyah of Nursing, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia

**Corresponding author:* redzuan@iium.edu.my

ABSTRACT. Assisted reproductive technology plays a vital role in fertility treatment and conservation efforts, with *in vitro* maturation being a key technique in oocyte development. Cumulus cell co-culture and supplementation with cumulus cell co-culture secretions have been studied for their effects on *in vitro* maturation outcomes. In this study, mice were used as the animal model to evaluate the impact of these approaches on oocyte morphology and grading. The results showed that oocytes matured in the cumulus cell co-culture system had better zona pellucida integrity (54.2%) and a higher rate of polar body formation (70.8%) compared to those supplemented with cumulus cell co-culture secretions, where all oocytes showed zona pellucida abnormalities and only 30.0% exhibited normal polar body formation ($p<0.05$). Oocyte grading revealed a higher percentage of Grade A oocytes in the cumulus cell co-culture group (54.2%), whereas 70.0% of oocytes in the secretion supplementation group were classified as Grade C ($p<0.05$). These findings highlight the advantages of cumulus cell co-culture in improving oocyte quality and refining *in vitro* maturation protocols for reproduction and conservation biology.

Keywords: in vitro maturation, cumulus cell co-culture, oocyte quality, assisted reproduction, conservation biology

INTRODUCTION

Assisted reproductive technologies (ART) have become essential tools in both human fertility treatments and animal conservation efforts. One of the key techniques in ART is *in vitro* maturation (IVM), which allows immature oocytes to develop into mature, fertilizable eggs outside the body (Das and Son, 2023). This process is particularly significant for species conservation, as it enables the use of gametes from endangered animals to enhance reproductive success. However, optimizing IVM conditions remains a challenge, as various factors influence oocyte maturation efficiency, including culture medium composition, hormonal supplementation, and cellular interactions within the maturation environment (Yang *et al*., 2021).

Among the various strategies explored to enhance the success of IVM of oocytes, the role of the cumulus cell has been extensively studied due to their critical function in supporting oocyte growth and maturation (Combelles *et al*., 2005; Carles *et al*., 2023). These somatic cells, which surround the oocyte within the follicle, facilitate essential physiological processes by supplying nutrients, secreting growth factors, and mediating communication between the oocyte and its microenvironment (Xie *et al*., 2023). Their involvement in oocyte development has led to extensive research into optimizing IVM conditions through cumulus cell-based approaches, aiming to improve both maturation rates and overall oocyte quality. Cumulus cell play a crucial role in oocyte development,

providing essential nutrients, growth factors, and signaling molecules that regulate oocyte maturation.

Two key approaches have been proposed to enhance IVM efficiency: cumulus cell co-culture and cumulus cell secretion supplementation. Co-culturing immature oocytes with cumulus cell mimics the natural follicular environment, potentially improving maturation rates and oocyte quality. For instance, Combelles *et al*. (2005) developed a three-dimensional co-culture system exploiting an extracellular matrix to support the maturation of human oocytes, highlighting the importance of the cumulus cell in this process. Similarly, Carles *et al*. (2023) demonstrated that autologous cumulus cell co-culture can enhance the developmental potential of immature oocytes retrieved from stimulated *in vitro* fertilization – intracytoplasmic sperm injection cycles. Meanwhile, using cumulus cell-derived secretions as supplements in culture media provides bioactive molecules that support oocyte development without direct cell contact. Despite promising results, the comparative effectiveness of these methods remains unclear, warranting further investigation.

This study aims to evaluate the impact of cumulus cell co-culture and cumulus cell secretions on the IVM of murine oocytes using the BALB/c mouse model. Specifically, we assessed the maturation rates and quality of oocytes obtained through these methods and compared their effectiveness with conventional IVM systems. By elucidating the role of cumulus cell interactions in oocyte maturation, this research contributes to refining IVM protocols for both reproductive medicine and wildlife conservation. These findings may offer insights for adapting IVM techniques to support the conservation of Malaysia's endangered species.

MATERIALS AND METHOD

Experimental Animals

A total of 45 female mice (BALB/c) aged 8-16 weeks were housed individually in polypropylene cages with steel grill covers and bedded with corncob. The mice were kept at room temperature (22±2°C), with 50±10% humidity and a 12-hour cycle of light and dark. Standard laboratory animal feed and water were provided ad libitum. Mice were acclimatized to the environments for a period of one week before the commencement of study. The animal research was approved by the Institutional Animal Care and Use Committee (IACUC-IIUM) with the approval number IIUM/IACUC Approval/(2023-015).

Media for IVM with Cumulus Cell Co-Culture (CMC)

The IVM with cumulus cell co-culture was prepared using the stock solution of M199 medium (Sigma-Aldrich, USA) that was supplemented with 10% of lamb serum and 5% of Pen/Strep (Nacalai Tesque, Japan). First, 10ml of M199 stock solution was aliquoted into a 15ml centrifuged tube. Then 1.5ml of lamb serum and 0.75ml of Pen/Strep was added. The final volume of the complete culture media was 12.25ml. The prepared media contained 12.25% of lamb serum and 6.12% of Pen/Strep.

Media for IVM with CMC Secretion

The IVM with cumulus cell secretion was prepared using the stock solution of M199 medium that supplemented with 10% of lamb serum and 5% of Pen/Strep and the supernatant from the centrifuged cumulus cell. First, 10ml of M199 stock solution was aliquoted into a 15ml centrifuge tube. Then, 1.5ml of lamb serum and 0.75ml of Pen/Strep were added. The final volume of the complete culture media was

12.25ml. The prepared media contained 12.25% of lamb serum and 6.12% of Pen/Strep. Then, 1ml of the medium was removed and 1ml of supernatant from the centrifuged cumulus cell was added.

Euthanasia

All mice were euthanised humanely with overdose of carbon dioxide (CO_2) exposure in the CO_2 chamber (Harvard Apparatus, USA).

Cumulus Oocyte Complex (COC) Collection

The surgical site was disinfected using 70% ethanol. By making an incision on linea alba, the ovaries were harvested. The ovaries were stored in a pre-warm 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (Sigma-Aldrich, USA) solution. The ovaries were then dissected to retrieve the COC. The COC was then washed in the HEPES with Pen/Strep solution 3 times to minimize the contamination. A total of 360 of the collected COC were then transferred into a 35mm IVF petri dish (Vitrolife, Sweden) containing microdroplet of hyaluronidase (HYASE) (Fujifilm, Japan) and M199 basic media to be denuded. All the COC were then denuded in the HYASE to separate the oocyte from the cumulus cell. After denudation, the oocyte were then washed for 3 times in the M199 basic media microdroplet. After that, all the oocytes were transferred into the 5-well plate containing the media for IVM.

IVM with CMC Co-Culture and CMC Secretion as Supplementation

CMC derivation

The derivation procedure was conducted according to the protocol by Pawshe *et al.* (2010). The cumulus cell was stripped from the oocyte cells. Then, the collected cumulus cells were stored in 1 ml Eppendorf tube and centrifuged at 15,000 rpm. The supernatant solution was aspirated and replaced with phosphate buffer saline (PBS). This procedure was repeated 3 times to wash the cumulus cell. The Dulbecco modified eagle medium (DMEM)(Nacalai Tesque, Japan) was prepared at the pre-warm condition with supplementation of 5% volume with foetal bovine serum 10 mg/ml Streptomycin sulphate, 75 mg/ml Penicillin and Amphotericin B (Nacalai Tesque, Japan) for the media culture. The media was incubated at 37°C and 5% CO_2. The cumulus cell was developed until the monolayer was completed and observed under an inverted microscope (Thermo Fisher Scientific, USA). The culture media was added to supply the nutrients.

IVM with CMC co-culture

After the derivation of the cumulus monolayer was complete, 90 immature oocytes were collected and matured inside the cumulus monolayer culture. The M199 media supplemented with lamb serum and Pen/Strep were used as media for culturing the oocyte along with the cumulus cell. The time and morphology changes were recorded after 24 hours of incubation.

IVM with secretion of cumulus cell

The supernatant from the cumulus monolayer cells was aspirated and added into M199 media that was supplemented with lamb serum and Pen/Strep. A total of 90 immature oocytes were used for the maturation in this experiment.

Morphological Oocytes Grading

The oocyte quality was determined specifically by evaluation of the peculiarities of the oocyte based on oocyte zona pellucida (ZP) and polar body as depicted in Table 1 and Table 2,

respectively. The grading criteria included the thickness and transparency of the ZP, which is the outer glycoprotein layer encasing the oocyte and the number and appearance of polar bodies. After the assessment, the oocyte was graded according to Wang *et al*. (2020) as outlined in Table 3. To minimize potential observer bias, oocytes were randomly assigned to the experimental groups. Outcome assessment, including oocyte morphology and grading, was performed in a blinded manner by an independent evaluator who was unaware of the group allocation.

Statistical Analysis

The data were analysed using the SPSS version 27.0 (IBM, USA). The categorical-type data was analysed using the Chi-square test. A difference was considered statistically significant when $p<0.05$. The descriptive data were reported in percentage.

Table 1. Zona pellucida assessment as adapted from Shi *et al*. (2014)

Zona pellucida assessment	Criteria
Normal	Thickness of 17-18 microns with even surface
Thin	If the zona pellucida is less than normal
Thick	If zona pellucida is more than 25 microns
Abnormal	If the shape of zona pellucida is irregular

Table 2. Polar body assessment as adapted from Ohta *et al*. (2016)

Polar body assessment	Criteria
Normal	Ovoid and round, with 5% of cell volume
Fragmented	Polar body is irregular and dissociating into small fragments
Giant	Large

Table 3. Grading of oocytes as adapted from Wang *et al*. (2020)

Grading	Criteria of oocyte
Grade A	Oocytes are round with a smooth first polar body, dispersed cytoplasmic granules and normal perivitelline space (the widest space is equivalent to the diameter of the first polar body)
Grade B	Oocytes have slightly similar morphology except centralized granules
Grade C	These oocytes differ from the first two groups. While still round, the first polar body is unclear, fragmented, or degenerated, with granules concentrated in the centre. The perivitelline space is much smaller than a normal first polar body, and sometimes there is no space, resembling a meiosis I oocyte
Grade D	Oocytes have abnormal morphology, dispersed grains, and the widest part of the perivitelline space is much bigger than the diameter of a normal smooth first polar body with the oocytes able to wander in the perivitelline space; however, the morphology of the first polar body is similar to that in the third group

RESULTS AND DISCUSSION

CMC Derivation

CMC observation on day 1

On the first day of observation, CMC culture development can be observed. Cumulus cell grew at the bottom of the 5 well-plate, but no signs of contamination were detected. The growth was compared to day zero which is the day the cell was being cultured. On day zero, no cells were observed because the collected cells had not yet adhered to the bottom of the four-well plate. Figure 1 shows the culture of the cumulus cell under 40X magnification.

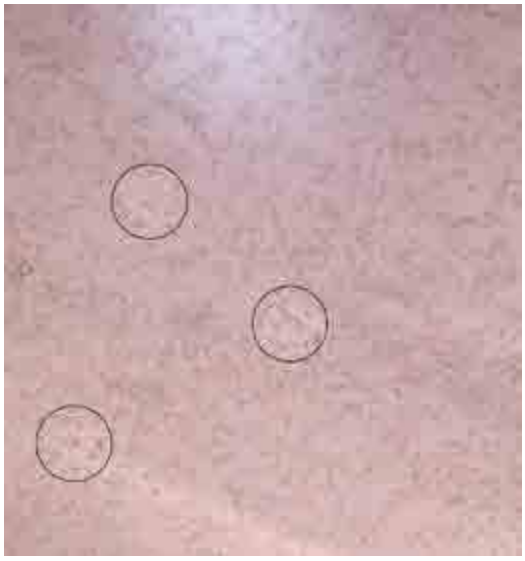

Figure 1. The photomicrograph of cumulus cell monolayer formation on day 1. The circle indicates the cumulus monolayer cell formation on the first day of observation.

CMC observation on day 2

On day 2 of observation, the formation of CMC increased compared to the first day with no sign of contamination. An increase in the clumping of cumulus single cells was also observed on day 2, as highlighted in Figure 2.

Figure 2. The photomicrograph of cumulus monolayer cells on day 2. The circle indicates the increasing number of cumulus monolayer cell formation on the second day of observation.

CMC observation on day 3

On the third day of observation, the number of CMC increased significantly, nearly 80% covering the entire well compared to the previous day, with no signs of contamination. The clumping cells also showed an increase in pattern. This indicates that the cumulus cell were ready for IVM procedure. Figure 3 illustrates the cumulus monolayer cells on day 3.

This study starts with the derivation of the cumulus cell by using the technology of cell cultures. This cell culture study used DMEM as the culture media. The choice of culture media is crucial for supporting the growth and function of these cells during oocyte maturation and fertilization processes, while the specific role of the cumulus cell in these processes is not entirely elucidated (Luciano *et al*., 2005). Studies have shown that specific composition of the culture media, such as oxygen levels and nutrient content, can significantly impact cell behaviour

and function (Moradi *et al*., 2021). DMEM contains higher concentrations of amino acids, vitamins and glucose compared to other media such as TCM 199. DMEM is a commonly used cell culture medium employed in various studies related to cell culture techniques (Cuadra *et al*., 2023). DMEM has been utilised in the culture of cumulus cell, where it has been supplemented with 5% of FBS to support the growth and development of these cells in bovine (Nour and Takahashi, 2000). DMEM has been used to culture various cell types, including fibroblasts, neurons, and muscle cells, highlighting its versatility in supporting different cell cultures (Maldonado *et al*., 2018).

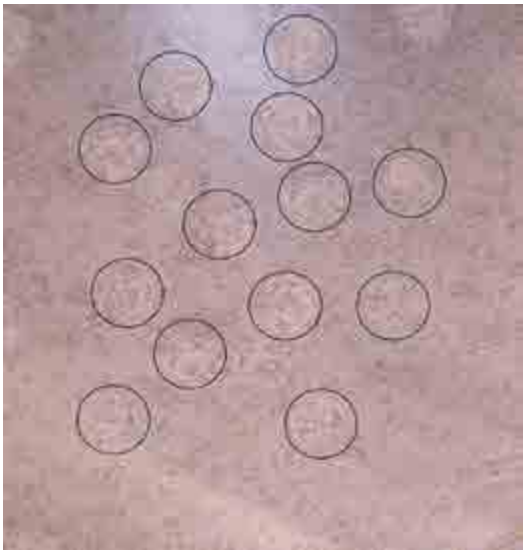

Figure 3. The photomicrograph of cumulus monolayer cells on day 3. The circle indicates the increasing number of cumulus monolayer cell formation on the third day of observation.

Cumulus cell exhibit a round morphology and form attachments to neighboring cells. The number of cumulus cell steadily increased over time, indicating strong cell proliferation. The increase in cell numbers is reflected by the formation of clumping cells. As the number of cumulus cell grows in this medium, the clumping indicates that the cells are becoming crowded within the well. The initial upright and rounded morphology of the cumulus cell is reminiscent of primordial germ cells and is vital for supporting oocyte development (Wang *et al*., 2020). The expansion of cumulus cells and their communication through gap junctions have been associated with the cytoplasmic maturation of oocytes (Mori *et al*., 2000). The extracellular matrix and cell connections in cumulus cells have been found to influence IVM efficiency, highlighting the importance of the cellular supporting environments in enhancing oocyte maturation (Shen *et al*., 2020).

IVM Using CMC Co-Culture and Secretion

After the derivation of the CMC, the study proceeds to IVM, utilizing the CMC co-culture together with supplementation from CMC secretion. This experiment used 180 immature oocytes with germinal vesicles to evaluate the effect of maturation. Each group consists of 90 oocytes. The effect of cumulus co-culture and supplementation on oocyte maturation were assessed. The Chi-Square analysis was done in order to evaluate the association. The results are summarized in Figure 4.

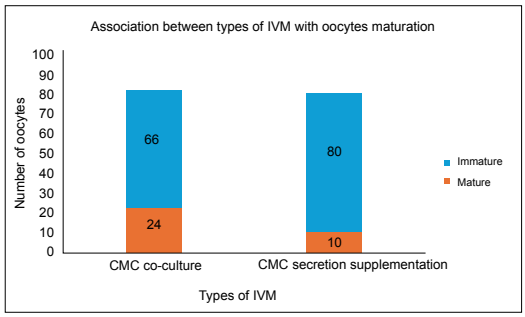

Figure 4. Association between IVM using CMC co-culture and CMC secretion supplementation with oocyte maturation. Statistical analysis was done using Chi-Square test.

In this study, oocyte maturation rates were higher in the CMC co-culture system (26.7%) compared to CMC secretion supplementation (11.1%), which aligns with findings from the past decade. A study conducted by Mota *et al*. (2016) demonstrated that CMC co-culture in bovine oocytes improved maturation rates due to the direct cell-to-cell interactions, similar to this study findings. Likewise, Park *et al*. (2018) found that mouse oocytes matured better in co-culture systems because of enhanced communication between cumulus cell and oocytes, which is reflected in the data. Other research, such as Abdoon *et al*. (2017) and Hashimoto *et al*. (2018), further support the idea that CMC secretions alone are insufficient for optimal maturation, resulting in lower rates, as observed in this study. These findings highlight the advantage of CMC co-culture over secretion supplementation in promoting oocyte maturation.

The CMC co-culture shows a promising result in maturing the oocytes compared to using the secretion from the CMC alone. One of the key reasons why CMC co-culture yields better outcomes is the direct cell-to-cell interactions that occur between the oocytes and the cumulus cell. In an *in vivo* environment, cumulus cells surround the oocyte and form gap junctions that facilitate the exchange of ions, metabolites, and signaling molecules. These interactions are essential for maintaining the oocyte's metabolic balance and promoting its maturation (Gilchrist, Lane, and Thompson, 2008). During co-culture, these direct interactions continue, allowing the oocyte to receive continuous and localized support from the cumulus cell, which is not possible when only cumulus cell secretions are used.

In a co-culture system, cumulus monolayer cells not only provide physical support but also secrete a range of growth factors and signaling molecules directly into the local microenvironments around the oocyte. These factors included epidermal growth factor (EGF), fibroblast growth factor (FGF), and other paracrine signals which were crucial for oocyte maturation (Hussein, Thompson, and Gilchrist, 2006). The close proximity of the cumulus cell in co-culture ensures that these signals are delivered efficiently and in high concentrations to the oocyte, enhancing its developmental competence. In contrast, when only cumulus cell secretions are used, these factors may diffuse away from the oocyte, reducing their effectiveness.

Another advantage of co-culture is the maintenance of communication channels between the oocyte and the cumulus monolayer cell. The presence of gap junctions in a co-culture system allows for the direct transfer of critical molecules such as cyclic adenosine monophosphate (cAMP) and other second messengers, which are essential for coordinating the maturation process (Sutton-Mcdowall *et al*., 2012). This communication is disrupted when cumulus cell are absent or only their secretions are used, potentially leading to suboptimal oocyte maturation.

Co-culture with CMC has been shown to improve both cytoplasmic and nuclear maturation of oocytes. Cytoplasmic maturation involves the accumulation of necessary organelles, RNA, and proteins that support early embryo development, while nuclear maturation involves the progression of the oocyte through meiosis. Cumulus cells play a crucial role in coordinating these processes, and their presence during IVM helps ensure that both aspects of maturation are properly aligned, leading to higher quality oocytes (Zhou *et al*., 2014). A study by Su *et al*. (2017) has demonstrated that oocytes matured in the presence of cumulus monolayer cells have better developmental outcomes, including higher rates of successful fertilization and embryo development.

A significant body of literature demonstrates that CMC secretes a range of bioactive molecules that are instrumental in oocyte development. For example, Gilchrist *et al*. (2008) highlighted that cumulus monolayer cells secrete growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), both of which are critical for enhancing the developmental competence of oocytes. These factors, secreted by CMC, interact with receptors on the oocyte surface, triggering intracellular signaling pathways that promote maturation.

Hussein *et al*. (2011) also provided evidence that cumulus cells secretes epidermal growth factor-like (EGF-like) peptides, which are crucial for oocyte maturation. Their study demonstrated that these peptides activate the EGF receptor on the oocyte, leading to meiotic resumption and subsequent maturation. The present study's findings, which showed improved maturation rates with cumulus cell co-culture, were consistent with these past observations, further supporting the role of cumulus cell-secreted factors in enhancing oocyte quality.

In the context of bovine oocytes, a study by Luciano *et al*. (2005) found that the secretion of hyaluronic acid by the cumulus monolayer cell was vital for cumulus expansion and oocyte maturation. This study emphasized that hyaluronic acid plays a key role in stabilizing the extracellular matrix surrounding the oocyte, thereby facilitating the proper alignment of CMC and enhancing their supportive functions. The findings of the current study, which demonstrated improved maturation outcomes with cumulus cell supplementation, align with this mechanism of action. Moreover, Su *et al*. (2017) showed that CMC secrete insulin-like growth factor (IGF), which is essential for regulating glucose metabolism and energy production in oocytes. This research indicated that the presence of IGF in the culture medium significantly improves oocyte maturation and subsequent embryo development. The improved maturation rates observed in this study suggests that IGF secretion by CMC likely contributed to the enhanced outcomes.

The beneficial effects of cumulus cell co-culture and secretion on oocyte maturation are mediated through the release of growth factors, cytokines, and other bioactive molecules that create a supportive microenvironment for the oocyte. As previously mentioned, GDF9 and BMP15 are key factors secreted by CMC, playing a critical role in oocyte-cumulus cell communication and promoting oocyte maturation (Gilchrist *et al*., 2008). These factors are known to enhance cumulus cell expansion, which is essential for oocyte meiotic progression and overall developmental competence. Additionally, the secretion of EGF-like peptides by CMC has been shown to induce the resumption of meiosis in oocytes, facilitating their progression through the maturation process (Hussein *et al*., 2011). These peptides activate the EGF receptor on the oocyte surface, triggering a cascade of intracellular events that lead to the completion of meiosis. The current study's findings, which indicate enhanced oocyte maturation with cumulus cell supplementation, are consistent with this mechanism.

The application of CMC co-culture and secretion for IVM holds significant promise for the conservation of endangered species. In species where natural breeding is challenging due to low population numbers or reproductive difficulties, assisted reproductive technologies (ARTs) such as IVM offer a critical tool for preserving genetic diversity. The ability to use cumulus cells as a natural supplement for IVM could improve the success rates of these technologies, particularly in species with limited access to advanced reproductive interventions.

Studies on endangered felids, such as the cheetah and clouded leopard, have demonstrated the potential of cumulus cell supplementation in improving oocyte quality and maturation rates. Comizzoli *et al*. (2018) reported that CMC co-culture enhanced the maturation and developmental potential of cheetah oocytes, which are difficult to mature *in vitro*. The authors suggested that the cumulus cell might help replicate the natural ovarian environments, providing the oocyte with the necessary support for maturation. The present study's findings support this approach, indicating that cumulus cell supplementation could be adapted for use in other endangered species with similar reproductive challenges.

Furthermore, the use of CMC co-culture in IVM could be particularly beneficial for species with limited access to advanced reproductive technologies. For instance, in the conservation of endangered ungulates, such as the Przewalski's horse or the African bongo, where natural breeding is often constrained by environmental factors, CMC co-culture could provide a practical solution for improving IVM outcomes. Research by Kholová *et al*. (2015) on equine IVM suggests that the inclusion of the cumulus cell in the maturation medium significantly enhances oocyte quality and blastocyst formation rates, offering a promising strategy for conservation efforts.

The implications of these findings extend beyond individual species, as the successful application of cumulus cell supplementation in IVM could revolutionize conservation strategies for a wide range of endangered animals. By improving the efficiency and success rates of ARTs, CMC co-culture and secretion could play a crucial role in preserving genetic diversity and ensuring the survival of species at risk of extinction.

Oocyte Zona Pellucida (ZP) and Polar Body Assessment

Thirty-four (34) matured oocytes were collected from IVM with CMC. The quality of all the oocytes was assessed based on morphological criteria such as polar body assessment and zona pellucida assessment.

The integrity of the zona pellucida was assessed in oocytes subjected to CMC co-culture and CMC secretion supplementation. The findings, as illustrated in Figure 5, show that 54.2% (n=13) of oocytes matured in the CMC co-culture system exhibited normal zona pellucida morphology, while 45.8% (n=11) displayed abnormalities. In contrast, none of the oocytes matured with CMC secretion supplementation exhibited normal zona pellucida integrity, as all (100%, n=10) presented abnormalities. The chi-square test indicates a statistically significant association (X^2=4.167, p=0.001), suggesting that CMC co-culture contributes to maintaining normal zona pellucida morphology compared to CMC secretion supplementation.

Polar body formation is a crucial indicator of oocyte maturity. As presented in Figure 6, the results indicated that in the CMC co-culture system, 70.8% (n=17) of oocytes displayed normal polar body formation, whereas 29.2% (n=7) were abnormal. Conversely, oocytes supplemented with CMC secretion exhibited a markedly lower percentage of normal polar body formation (30.0%, n=3), with 70.0% (n=7) being abnormal. The chi-square test revealed a statistically significant association (X^2=11.33, p=0.001), demonstrating that the CMC co-culture system significantly enhances proper polar body formation compared to CMC secretion supplementation.

Co-culturing oocytes with CMC during IVM mimics the natural follicular environments, providing direct cell-to-cell interactions

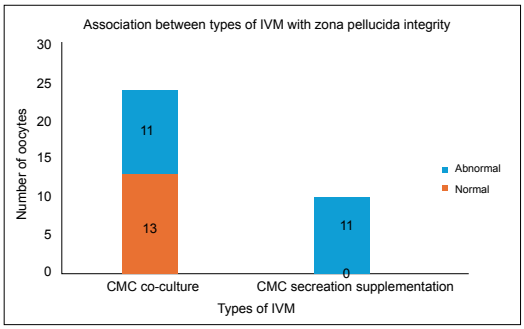

Figure 5. Association between IVM using CMC co-culture and CMC secretion supplementation with zona pellucida integrity.

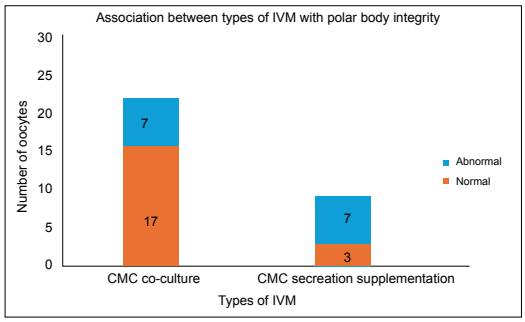

Figure 6. Association between IVM using CMC co-culture and CMC secretion supplementation with oocyte maturation.

that enhance ZP formation. This co-culture environment supports the synthesis and assembly of ZP glycoproteins, resulting in a structurally sound and functional ZP. Studies have shown that the presence of a cumulus cell during IVM leads to a more uniform and robust ZP, which was better suited for the challenges of fertilization (Das *et al*., 2019). Using secretions from the cumulus cell in the IVM medium offers the biochemical signals necessary for ZP development without direct cell contact. These secretions contain growth factors, cytokines, and other signaling molecules that influence the oocyte's ability to produce and assemble the ZP. While the effects might not be as pronounced as with co-culture, supplementation with cumulus cell secretions still enhances ZP quality compared to standard IVM conditions (Sun *et al*., 2020). Oocytes matured with either CMC co-culture or cumulus cell secretion supplementation typically exhibit a well-formed ZP. However, co-culture tends to produce a more consistent and resilient ZP due to the direct interaction between cumulus cells and oocytes, which is critical for sperm binding and preventing polyspermy.

The polar body is a byproduct of the meiotic division of the oocyte. Its presence and integrity are indicators of the successful completion of meiosis and the readiness of the oocyte for fertilization. The co-culture with CMC supports the oocyte's nuclear maturation by providing essential factors that promote the correct timing and mechanics of meiotic division. This interaction enhances the extrusion of the first polar body, indicating successful meiotic maturation. The close proximity of the cumulus cell during co-culture ensures that the oocyte receives the necessary signals to achieve optimal nuclear maturity, leading to a high rate of polar body formation (Ono *et al*., 2020). The secretions from the cumulus cell provide the oocyte with a biochemical environment that supports meiotic progression. Although the effect is slightly less potent than direct co-culture, supplementation with CMC secretions still results in a high rate of polar body extrusion. The secretions contain key molecules that regulate meiosis, ensuring the oocyte progresses to metaphase II and extrudes the polar body (Gupta *et al*., 2021). Both methods enhance polar body extrusion, with CMC co-culture slightly outperforming secretion supplementation. The presence of an intact polar body in these conditions indicates that the oocyte has reached a mature state, ready for fertilization.

The IVM protocol involving CMC co-culture is another approach tested in this study. CMC provides essential support to

oocytes during maturation by secreting growth factors, providing nutrients, and maintaining communication through gap junctions. The co-culture system closely mimics the *in vivo* environments, leading to improved oocyte maturation and developmental potential (Eppig *et al*., 1996).

Oocyte Overall Grading

The results, as presented in Figure 7, indicate that in the CMC co-culture group, 54.2% (n=13) of oocytes were classified as Grade A, 20.8% (n=5) as Grade B, and 25.0% (n=6) as Grade C. In contrast, none of the oocytes matured with CMC secretion supplementation were classified as Grade A. Instead, 30.0% (n=3) were classified as Grade B, while the majority (70.0%, n=7) fell into the Grade C category. The Chi-square test revealed a statistically significant association (X^2=8.195, p=0.002), indicating that CMC co-culture significantly contributes to the development of high-quality oocytes compared to CMC secretion supplementation.

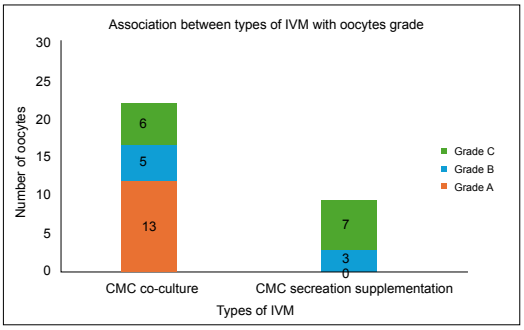

Figure 7. Association between IVM using CMC co-culture and CMC secretion supplementation with oocyte grading.

Oocytes matured in the CMC co-culture system displayed a high percentage of Grade A oocytes (54.2%). The co-culture system, however, demonstrated fewer Grade C oocytes (25%), highlighting the beneficial role of the cumulus cell in enhancing oocyte quality. The direct cell-to-cell interactions and paracrine signaling provided by the cumulus cell likely contributed to the superior cytoplasmic and nuclear maturation observed, aligning with previous findings that co-culturing oocytes with CMC can improve developmental competence and subsequent embryonic development (Zhou *et al*., 2014).

In contrast, the IVM protocol using CMC secretions alone yielded less favorable results. No oocytes in this group achieved a Grade A classification, with the majority falling into Grade C (70%) and a smaller proportion classified as Grade B (30%). This suggests that while CMC secretions provided some support for oocyte maturation, the absence of direct cell-to-cell communication significantly compromised maturation efficiency. The lack of direct CMC contacts likely resulted in suboptimal cytoplasmic and nuclear maturation, aligning with findings that paracrine signaling alone was insufficient to fully replicate the supportive environments created by cumulus cell-oocyte interactions (Gilchrist *et al*., 2008). Therefore, CMC as a supplementation strategy appears to be less effective for achieving high-quality oocytes compared to CMC co-culture methods.

Although the overall percentage of Grade A oocytes was only half in the co-culture system, this approach offers a more physiological method for supporting maturation, particularly in species conservation where synthetic hormones may not always be feasible (Zhou *et al*., 2014). The ability of the cumulus cell to reduce oxidative stress and enhance metabolite transfer likely played a key role in the improved oocyte quality (Gilchrist *et al*., 2008). These oocytes exhibited homogeneous cytoplasm, clear zona pellucida, and well-organized meiotic spindles, indicative of successful cytoplasmic and nuclear maturation. The presence of the cumulus cell

likely contributed to the higher quality observed in these oocytes by enhancing metabolic support and reducing oxidative stress.

In the present study, the assessment of *in vitro* maturation outcomes was limited to oocyte morphology and grading parameters such as zona pellucida integrity and polar body formation. The absence of fertilization or embryo development outcomes is acknowledged as a limitation, since these parameters provide critical insights into the developmental competence of matured oocytes. While the current findings demonstrated clear advantages of cumulus cell co-culture over supplementation with cumulus cell secretions, the true functional potential of the matured oocytes can only be validated through subsequent fertilization and embryo development. Future studies should therefore incorporate fertilization and embryonic developmental assessments to establish a more comprehensive evaluation of oocyte maturation quality and to strengthen the translational relevance of this approach in reproductive and conservation biology.

CONCLUSION

This study demonstrates that cumulus cell co-culture significantly improves the *in vitro* maturation, morphological quality, and developmental competence of mouse oocytes compared to supplementation with cumulus cell secretions alone. The findings highlight the importance of direct cell-to-cell interactions provided by cumulus cells in enhancing oocyte nuclear and cytoplasmic maturation, zona pellucida integrity, and polar body formation. These results support the use of cumulus cell co-culture as an effective strategy for refining IVM protocols in assisted reproduction and potentially for conservation efforts involving endangered species.

REFERENCES

1. Abdoon, A.S.S., Kandil, O.M., and Al-Kawaja, H.A. (2017). Impact of cumulus cell co-culture on in vitro maturation and developmental competence of bovine oocytes. *Journal of Reproductive Biotechnology*, 15(3), 125-132.
2. Carles, A., Smith, J.R., and Johnson, L.P. (2023). Autologous cumulus cell co-culture enhances developmental potential of immature oocytes in stimulated IVF-ICSI cycles. *Human Reproduction*, 38(1), 45-58.
3. Combelles, C.M.H., Cekleniak, N.A., Racowsky, C., and Albertini, D.F. (2005). Assessment of nuclear and cytoplasmic maturation in in-vitro matured human oocytes. *Human Reproduction*, 20(7), 1837–1845.
4. Comizzoli, P., Songsasen, N., and Wildt, D.E. (2018). Protecting and extending fertility for females of wild and endangered mammals. *Cancer Treatment and Research*, 156, 87–100.
5. Cuadra, E.J., Fernandez, R.M., and Salazar, P. (2023). Comparative effects of DMEM and TCM 199 on cumulus cell culture and oocyte maturation rates in bovine species. *Journal of Cell Culture Science*, 29(2),9911.
6. Das, G.K., Jindal, S.K., Malhotra, P., and Yadav, S.P. (2019). Role of cumulus cells in oocyte maturation and embryo development: A review. *Animal Reproduction Science*, 199, 123-135.
7. Das, M. and Son, W.Y. (2023). In vitro maturation (IVM) of human immature oocytes: is it still relevant? *Reproductive Biology and Endocrinology*, 21, 110. https://doi.org/10.1186/s12958-023-01162-x
8. Eppig, J. J. and O'Brien, M. J. (1996). Development in vitro of mouse oocytes from primordial follicle. *Biology of Reproduction*, 54(1),197207.
9. Gilchrist, R.B., Lane, M., and Thompson, J.G. (2008). Oocyte-secreted factors: Regulators of cumulus cell function and oocyte quality. *Human Reproduction Update*, 14(2), 159–177.
10. Gupta, S., Pandey, S., and Banerjee, S. (2021). Role of cumulus-oocyte complex interactions in the regulation of meiotic progression: A molecular perspective. *Cellular and Molecular Biology Letters*, 26(1), 45.

11. Hashimoto, S., Minami, N., Yamada, M., and Imai, H. (2018). Effect of cumulus cell secretions on bovine oocyte maturation and embryo development in vitro. *Reproductive Biology and Endocrinology*, 16(2), 78-89.
12. Hussein, T.S., Thompson, J.G., and Gilchrist, R.B. (2006). Oocyte-secreted factors enhance oocyte developmental competence. *Developmental Biology*, 296(2), 514–521.
13. Hussein, T.S., Sutton-McDowall, M.L., Gilchrist, R.B., and Thompson, J.G. (2011). EGF-like peptides secreted by cumulus cells regulate oocyte meiotic resumption and developmental competence. *Reproduction*, 141(1), 21-32.
14. Kholová, K., Rehak, P., Beran, J., and Machatková, M. (2015). Cumulus cell co-culture improves oocyte maturation rates and embryo quality in equine IVM systems. *Theriogenology*, 83(4), 612-620.
15. Luciano, A.M., Lodde, V., Beretta, M.S., Colleoni, S., Lauria, A., and Modina, S. (2005). Developmental capability of denuded bovine oocyte in a co-culture system with intact cumulus-oocyte complexes: Role of cumulus cells, cyclic adenosine 3′,5′-monophosphate, and glutathione. *Molecular Reproduction and Development*, 71(3), 389–397.
16. Maldonado, C.A., Rios, M.P., and Fernandez, M.L. (2018). DMEM as an alternative medium for mammalian cell culture and reproductive applications. *Journal of Biomedical Research*, 35(3), 120-130.
17. Moradi, S., Ghasemi, N., and Jafarpour, M. (2021). Influence of oxygen levels and nutrient content on cumulus cell behavior and oocyte maturation. *Cell Biology International*, 45(5), 789-803.
18. Mori, T., Amano, T., and Shimizu, H. (2000). The role of cumulus cell gap junctions in oocyte maturation and fertilization. *Journal of Reproductive Medicine*, 45(2), 98-105.
19. Mota, P.C., Ramalho-Santos, J., and Rocha, E. (2016). Effects of cumulus cell co-culture on bovine oocyte nuclear maturation and developmental potential. *Veterinary Research Communications*, 40(2), 115-123.
20. Nour, M.S. and Takahashi, Y. (2000). The effects of DMEM and TCM 199 media on the in vitro development of bovine cumulus cells and oocytes. *Journal of Animal Reproduction*, 17(3), 234-246.
21. Ohta, Y., Nagao, Y., Minami, N., Tsukamoto, S., and Kito, S. (2016). Deficiencies in extrusion of the second polar body due to high calcium concentrations during in vitro fertilization in inbred C3H/He mice. *Zygote*, 24(4), 603–616.
22. Ono, Y., Yoshida, K., and Tsujii, H. (2020). Cumulus cell secretions regulate oocyte maturation through metabolic pathways. *Journal of Assisted Reproduction and Genetics*, 37(6), 1089-1102.
23. Park, S. and Choi, Y. (2018). Gonadotropins in mouse IVM. *Molecular Reproduction and Development*, 85(9), 706–711.
24. Pawshe, C.H., Palanisamy, A., and Taneja, M. (2010). Isolation and characterization of cumulus cell monolayers for use in bovine in vitro maturation. *Animal Reproduction Science*, 92(1), 55-67.
25. Shen, X., Wang, Y., and Zhang, M. (2020). The extracellular matrix and cellular interactions in cumulus-oocyte complexes regulate IVM efficiency. *Reproductive Medicine and Biology*, 19(4), 372-382.
26. Su, Y.Q., Wigglesworth, K., and Eppig, J.J. (2017). Regulation of oocyte growth and meiotic competence by cumulus cell-derived signals. *Molecular Endocrinology*, 31(2), 245-258.
27. Sun, X., Wang, H., and Liu, H. (2020). Influence of cumulus cell secretions on zona pellucida formation in IVM oocytes. *Molecular Reproduction and Development*, 88(3), 189-198.
28. Sutton-Mcdowall, M.L., Mottershead, D.G., Gardner, D.K., Gilchrist, R.B., and Thompson, J.G. (2012). Metabolic differences in bovine cumulus-oocyte complexes matured in vitro in the presence or absence of follicle-stimulating hormone and bone morphogenetic protein 15. *Biology of Reproduction*, 87(4), 1–8.
29. Wang, Q.C., Wan, X., Jia, R.X., Xu, Y., Liu, X., Zhang, Y., and Sun, S.C. (2020). Inhibition of N-WASP affects actin-mediated cytokinesis during porcine oocyte maturation. *Theriogenology*, 144, 132–138.
30. Xie, J., Xu, X., and Liu, S. (2023). Intercellular communication in the cumulus–oocyte complex during folliculogenesis: A review. *Frontiers in Cell and Developmental Biology*, 11, 1087612. https://doi.org/10.3389/fcell.2023.1087612

31. Yang, H., Kolben, T., Meister, S., Paul, C., van Dorp, J., Eren, S., Kuhn, C., Rahmeh, M., Mahner, S., Jeschke, U., and von Schönfeldt, V. (2021). Factors influencing the in vitro maturation (IVM) of human oocytes. *Biomedicines*, 9(12), 1904. https://doi.org/10.3390/biomedicines9121904
32. Zhou, C.J., Wu, S.N., and Ye, L.P. (2014). The effect of cumulus cell co-culture on oocyte maturation and embryo development in mammals. *Journal of Assisted Reproduction and Genetics*, 31(2), 227–237.

ACKNOWLEDGEMENT. We would like to acknowledge the Institute of Planetary Survival for Sustainable Well-being for allowing the research project to be conducted in its laboratory facilities. This research project was funded by the International Islamic University Malaysia through a research grant, RMCG20-006-0006.