*Pakistan J. Zool., pp 1-16, 2024.* **DOI**: https://dx.doi.org/10.17582/journal.pjz/20240214054643

Review Article

# Cytoplasmic and Nuclear Maturation of Intact and Reconstructed Oocytes Controlling the Developmental Competence of Embryos

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**Article Information**
**Received 14 February 2024**
**Revised 06 May 2024**
**Accepted 11 May 2024**
**Available online 16 October 2024**
**(early access)**

**Authors' Contribution**
**AAM and SA designed the study and wrote the manuscript. IA collected the data. SA and WA analyzed the results and wrote the manuscript. AM and AM prepared figures. All authors revised the manuscript, and approved the final version.**

**Key words**
**Follicle, Cumulus, Cytoplast, Nucleus, Nucleolus, Maturation, Embryo**

## ABSTRACT

Oocytes grow and develop within several distinct ovarian follicles during the fetal and postnatal periods, and they ovulate in each estrous or menstrual cycle from ovarian follicles after puberty. Ovarian follicles start to form when oogonia are surrounded by a single layer of pregranulosa follicular cells and are called primordial follicles. Primordial follicles grow and develop into further stages upon activation. The growth and development of primordial follicles to pre-ovulatory follicles in mammals lasts several months. Oocytes acquire developmental competence during the preantral and antral ovarian follicle stages. The final maturation stage of ovarian follicles occurs during estrous or menstrual cycles. Changes occur in the cytoplasts, nuclei, and the surrounding follicular cells of oocytes for completion of meiotic maturation, fertilization, and further embryo development either *in vivo* or *in vitro*. The cytoplasmic maturation of oocytes helps to surpass the maternal zygotic block and progress for further embryo development. Hence, the cytoplasts of enucleated germinal vesicle (GV) or mature (metaphase II; MII) oocytes control development after embryonic/somatic nuclear transfer. Therefore, this review was conducted to collect and consolidate the current knowledge of cytoplast and nuclear material effects on maturation of intact and reconstructed oocytes and further developmental competence of embryos *in vivo* and *in vitro* and their importance for nucleus reprogramming and fertility.



## INTRODUCTION

Oocytes grow and develop during fetal and postnatal periods within several distinct ovarian follicles, and they ovulate from the ovarian follicles after puberty in each estrous or menstrual cycle. The formation of ovarian follicle begins with primordial follicles, where oogonia are surrounded by pregranulosa cells (O'Connell and Pepling, 2021; Mohammed *et al*., 2022). The growth and development of primordial follicles occur upon activation of pregranulosa cells to reach the preantral and antral follicle stages (Zhang *et al*., 2014; Wu *et al*., 2021) (Fig. 1).

The changes that occur during follicle development include granulosa cell proliferation, oocyte growth, and theca cell differentiation (Richards and Pangas, 2010). Preantral follicle growth is gonadotropin-independent, whereas antral follicle growth is gonadotropin-dependent (Chakravarthi *et al*., 2021). Tang *et al*. (2012) investigated the role of follicle stimulating hormone (FSH) in combination with growth and differentiation factor-9 or basic fibroblast growth factor on the survival, activation, and growth of bovine primordial follicles, which improved after long-term culture of ovarian tissue.

Most of the information on ovarian follicle growth and development stems from studies on ruminants, swine,

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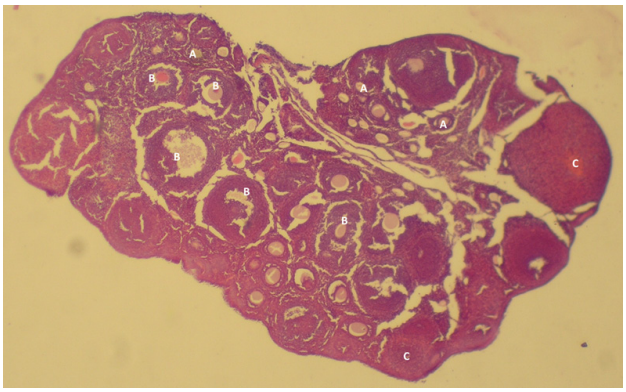

Fig. 1. Ovarian follicle structures upon fallopian tube ligation in mice; preantral follicles (A), antral follicles (B) and corpora lutea (C) structures. N.B. Differences in sizes of oocytes and the layers of surrounding cells inside the follicles.

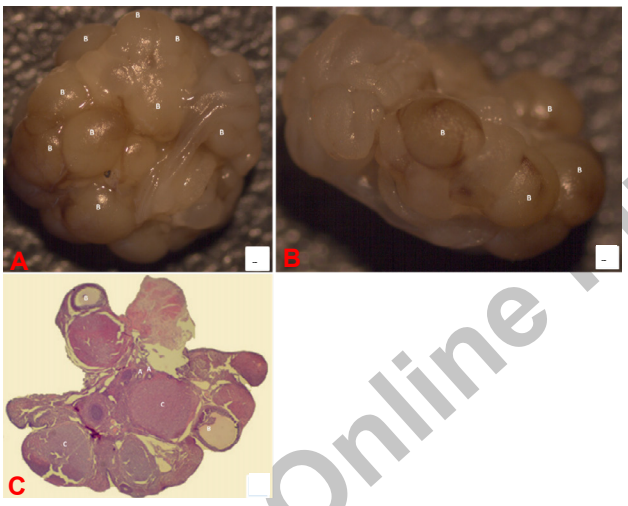

Fig. 2. The structures of ovarian follicle in control (A) and ovarian transplanted tissues in rats (B, C). The images show preantral follicles (A), antral follicles (B), and corpora lutea (C).

rodent species, and humans (Senosy *et al*., 2017; Bunel *et al*., 2020; Baerwald and Pierson, 2020; Pournaghi *et al*., 2021; Ko *et al*., 2021). Several factors such as species (Gordon, 2003; Patton *et al*., 2021), nutrition and feed additives (Mohammed and Attaai, 2011; Fernandes *et al*., 2016; Senosy *et al*., 2017, 2018; Mohammed 2018, 2019; Liang *et al*., 2012; Moulavi and Hosseini, 2019; Gutiérrez-Añez *et al*., 2021; Saini *et al*., 2022; Al-Mufarji *et al*., 2022; Al-Masruri *et al*., 2022a, b) and males (Taira *et al*., 2022) have effect on ovarian follicle growth and development. In addition, recently adapted techniques of ovarian synchronization, ovarian superovulation, and ovarian transplantation for dysfunction treatment and the maximizing of reproduction are known to change ovarian follicle growth and the development of the oocytes they contain (Gordon, 2003; Ata and Telek, 2021) (Fig. 2). Superovulation protocol via gonadotrophin stimulation might result in abnormal changes in cytoplast and nucleus structure, lower the concentration of estradiol hormone, and disrupt cumulus cell mRNA (Algriany *et al*., 2007; von Wolff *et al*., 2022). Moreover, Kanitz *et al*. (2002) found that high doses of FSH could lead to defects in ovulation at the level of the pituitary gland and ovaries. Consequently, the transfer of GV nuclei, nucleoli, or cytoplasts of highly competent oocytes to abnormal oocytes through adapted techniques may treat abnormalities in oocytes.

Oocytes acquire developmental competence during ovarian follicle development through changes in the nucleus and cytoplast. Therefore, the recipient cytoplast and donor nucleus stages of artificial gametes affect developmental competence. Such artificial gametes may be used for different purposes, such as studying of embryonic/somatic nucleus reprogramming in recipient cytoplasts, production of cloned and transgenic animals, and treatment of infertility (Campbell *et al*., 1996; Wilmut *et al*., 1997; Mohammed *et al*., 2019; Singh *et al*., 2022; Dolma *et al*., 2022). The roles of cytoplasmic maturation and nuclear progression to the MII stage of intact and reconstructed oocytes in controlling developmental competence of oocytes to embryos are compiled and discussed in this review.

## ASPIRATION AND DEVELOPMENTAL COMPETENCE OF CUMULUS-ENCLOSED GERMINAL VESICLE OOCYTE

Cumulus-enclosed germinal vesicle oocytes (COCs) can be aspirated or picked-up (OPU) from different animals, humans, or slaughterhouse ovaries (Mohammed *et al*., 2005; Jin *et al*., 2016; Ongaratto *et al*., 2020; Girsh, 2021; Al-Zeidi *et al*., 2022a, b) for *in vitro* embryo production. The OPU approach has been applied in humans to overcome abnormalities such as hidden ovaries, severe tubal diseases, or multiple adhesions with a success rate lower than 50%. Oocytes are picked up from genetically superior animals before puberty, early gestation, and early postpartum through laparoscopic and ultrasonic methods to shorten the interval between generations and increase animal production (Ongaratto *et al*., 2020; Furukawa *et al*., 2021). COCs have been used for *in vitro* embryo production through oocyte maturation (IVM), fertilization or activation (IVF), and culture (IVC) of different animal species and humans. The development of COCs to blastocyst stage does not exceed 30-40% (Mohammed

*et al*., 2005; Somfai *et al*., 2012). Several factors, such as ovary storage time, wave of estrous cycle, follicle diameter, oocyte quality, incubation time, maturation environment, nutrition and feed additives, and male effect, affect the developmental competence of aspirated oocytes to embryos (Schwartz *et al*., 1998; Demyda-Peyrás *et al*., 2013; Senosy *et al*., 2017, 2018; Taira *et al*., 2022) (Table I).

The development of oocytes to embryos increases when slaughterhouse ovaries are exposed to warm conditions for a few h before oocyte aspiration. Storage of ovaries at 30 °C for 3-4 h after slaughter has led to an increase in the number of oocytes developed to embryos. It was suggested that a storage period of 4 h resulted in the formation of an environment within the 3-8 mm follicles, similar to that which occurs in preovulatory follicles. In our study, changes in follicular fluid (FF) composition at times of zero, 4, and 8 h in goat ovaries were investigated. The most notable changes in FF composition were the concentrations of glucose, cholesterol, and triglycerides, which are sources of energy for oocytes. This might partially explain the increase in the developmental competence of oocytes collected from the ovaries 4 h after slaughter but decrease thereafter.

**Table I. Factors affecting developmental competence of aspirated oocytes *in vitro*.**

| Ovaries | Treatments | Effects | References |
|---|---|---|---|
| Slaughter-house ovaries | Storage the ovaries 30 °C for 3-4 h | Increase developmental competence of oocytes to embryos | Luu *et al*., 2011 |
| | Defined and undefined media | Variable effects on oocyte maturation and embryo development | Mohammed *et al*., 2005; Madkour *et al*., 2016; Spacek and Carnevale, 2018; Abdoon *et al*., 2018 |
| | IGF-1 and FF | Variable effects on oocyte maturation and embryo development | Oberlender *et al*., 2013 |
| | Fetal calf serum | Increase developmental competence of oocytes to embryos | Mohammed *et al*., 2005; Moulavi and Hosseini, 2019 |
| | Follicular fluid | Variable effects on oocyte maturation and embryo development depending on follicle sizes collected and added percentage to the maturation medium | Mohammed *et al*., 2005; Sinclair *et al*., 2008 |
| | Co-culture cumulus and oviduct cells | Increase developmental competence of oocytes to embryos | Lee *et al*., 2018 |
| | Cumulus cells surrounding the oocytes | Increase developmental competence of oocytes to embryos | Mohammed 2006; Mohammed *et al*., 2005, 2008, 2010, 2019; Al-Zeidi *et al*., 2022 |
| | Hormone | Increase developmental competence of oocytes to embryos | Moulavi and Hosseini, 2019 |
| | Follicule size | Variable effects where the increase of follicle sizes enhance developmental competence of oocytes to embryos | Shabankareh *et al*., 2014; Gordon, 2003; Maylinda *et al*., 2018 |
| Live organisms | Follicule size | Variable effects on oocyte maturation and embryo development where the higher follicle size the higher developmental competence of oocyte to embryos | Hasler *et al*., 1998; Maylinda *et al*., 2018; Raj *et al*., 2018 |
| | Follicular wave | Variable effects on oocyte maturation and embryo development | Gordon *et al*., 2003; Cavalieri *et al*., 2018; Baby and Bartlewski, 2011 |
| | Follicular and luteal stages | Variable effects on oocyte maturation and embryo development | Gordon *et al*., 2003 |
| | Nutrition | Variable effects on oocyte maturation and embryo development | Gordon *et al*., 2003 |
| | Feed additives | Variable effects on oocyte maturation and embryo development | Senosy *et al*., 2017, 2018; Mohammed and Al-Hozab, 2018 |

The more cumulus cells surround the oocytes and homogeneity of the oocytes cytoplasts, the greater the developmental competence of oocytes to embryos (Mohammed *et al*., 2005). In addition, the male effect stimulation of super ovulated females during the last phase of the FSH regimen increase the number and diameter of large follicles, in addition to the number of corpora lutea and viable embryos, compared to unstimulated ewes (Taira *et al*., 2022). Therefore, these factors should be taken into consideration for enhancement of oocyte quality, oocyte maturation, fertilization, and development of embryos.

## OOCYTE QUALITY AND GENE EXPRESSION

Oocyte quality is critical for maturation, fertilization, and embryonic and fetal development. The higher the oocyte quality, the higher the developmental competence of the resulting embryo and fetus. Oocytes are graded morphologically according to the cumulus cells surrounding the oocytes, diameter of the oocyte, and homogeneity of the cytoplasts (Mohammed *et al*., 2005; Lopes *et al*., 2010; Al-Zeidi *et al*., 2022). Castaneda *et al*. (2013) found that both active mitochondria and lipid content were correlated with oocyte diameter. In addition, brilliant cresyl blue staining (BCB) were used to select developmentally competent GV oocytes of different species (Opiela and Kątska-Książkiewicz, 2013; Jewgenow *et al*., 2019; Mohammed and Al-Hozab, 2020). Bovine GV oocytes stained with +BCB had a relatively higher developmental competence than non-stained BCB oocytes. A previous study investigated the relationship between BCB staining, lipid content, and active mitochondria. They concluded that higher lipid content of bovine oocytes stained with BCB might provide higher developmental competence. In addition, Sinclair *et al*. (2008) investigated the amino and fatty acid content in the follicular fluid of bovine follicles as a predictor of embryo development. They found that two amino acids (alanine and glycine) had the highest value for predicting early embryonic development, and this might serve as a useful tool in measuring COCs quality.

In recent years, transcriptome and proteome have been substantially explored as biomarkers for oocyte quality owing to the progression of the new technologies. The approach is an invaluable tool for clinical practice in assisted reproductive technologies, obstetrics, and gynecology (Li *et al*., 2019). The expression patterns of glucose metabolism genes (G6PDH, LDH, and PDH) were investigated in buffalo oocytes matured under different glucose concentrations (Kumar *et al*., 2013). The pattern of G6PDH expression during oocyte maturation and early embryonic development is predictive of oocyte quality and developmental competence of embryos. However, Liu *et al*. (2021) found in good prognosis IVF patients that mitochondrial DNA copy number of cumulus cells is not linked to embryo implantation. Therefore, further studies on gene expression are required to determine the relation between oocyte quality and further development of oocytes into preimplantation and post implantation embryo stages.

## NUCLEAR AND CYTOPLASMIC MATURATION OF INTACT OOCYTES

Oocytes require several months to acquire developmental competence before maturation (Blondin *et al*., 1997; Hashimoto *et al*., 2002). Small follicles contain inhibiting maturation substances within the follicles, which disappear in the preovulatory follicle simultaneously with the LH surge. Therefore, oocytes resume the first meiotic division to the metaphase II (MII) stage in vivo after the LH surge and *in vitro* when they are removed from antral follicles and cultured under favorable *in vitro* conditions (38.5 °C, 90% humidity, 5.0% $CO_2$).

Oocyte maturation *in vitro* was described nearly 85 years ago when rabbit and human oocytes were aspirated from follicles and cultured in a favorable medium (Pincus and Enzmann, 1935). It has been indicated that the developmental competence of *in-vitro* matured oocytes is lower than that of *in-vivo* matured oocytes (Leibfried-Rutledge *et al*., 1987; Margalit *et al*., 2019; Sakaguchi and Nagano, 2020) due to insufficient cytoplasmic maturity (Blondin *et al*., 1997). The longer the maturation time of oocytes, the lower the developmental competence of the resulting embryos, as indicated in swine and camel oocytes, compared to oocytes of other species (Wani and Hong, 2020; Mohammed and Al-Hozab, 2020; Li *et al*., 2022).

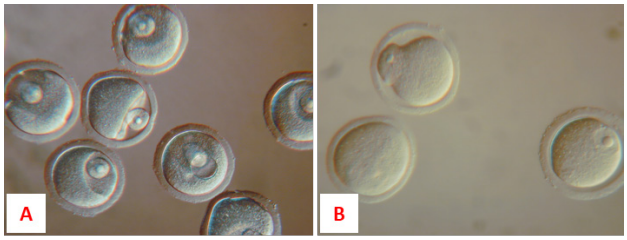


Fig. 3. The germinal vesicle karyoplasts placed under the zona pellucida of enucleated germinal vesicle oocytes (A) and fused with cytoplasts (B).

The morphological maturation stages of intact GV or reconstructed GV oocytes (Figs. 3 and 4) include oocyte nuclear and cytoplasmic maturation, in addition to cumulus expansion of the surrounding cells (Fig. 4). The germinal

vesicle of denuded oocytes is visible in rodent, rabbit, and human oocytes, whereas it is not visible in ruminant oocytes because of the presence of lipid droplets in the cytoplasm. Gordon (2003) described the chronology of events during bovine oocyte maturation as inductive and synthetic phases. The nuclear changes included germinal vesicle breakdown (GVBD), which occurred at 2-3 h in rodent oocytes and 6-8 h in ruminant oocytes, respectively. Rodent oocytes reached the MI stage at 11-13 h and MII stage at 15-17 h. The ruminant oocytes reached the MI stage at 15-21 h and MII stage at 24 h (Gordon, 2003; Virant-Klun *et al*., 2018; Mohammed *et al*., 2008, 2010, 2019).

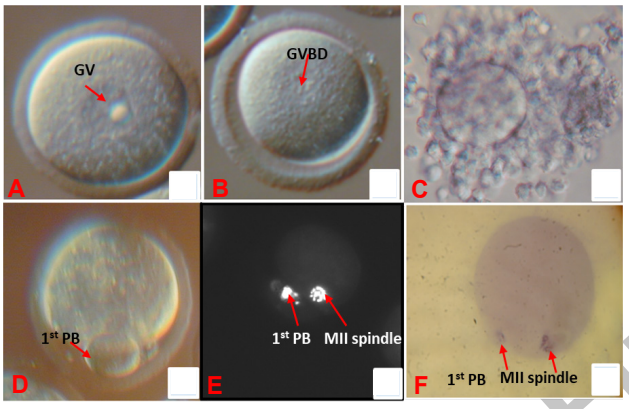


Fig. 4. Oocyte maturation; denuded germinal vesicle oocyte (A), germinal vesicle breakdown (B), cumulus expansion (C), polar body extrusion (D), fluorescence staining of matured oocyte (E), hematoxylin stain of matured oocyte (F).

Follicle size, follicular wave during the estrous cycle, follicular and luteal stage of estrous, species and age (Gordon, 2003; Patton *et al*., 2021), nutrition and feed additives (Cavalieri *et al*., 2018; Mohammed, 2018, 2019; Mohammed and Al-Hozab, 2020; Liang *et al*., 2012; Moulavi and Hosseini, 2019; Pournaghi *et al*., 2021; Gutiérrez-Añez *et al*., 2021; Saini *et al*., 2022), and male effects (Taira *et al*., 2022) were found to affect oocyte maturation. In addition, super stimulation of ovarian follicles via gonadotropin injections resulted in changes in small, medium, and large follicles according to the number and dose of gonadotropin injections and side of the ovary (Abdelnaby *et al*., 2021). FSH stimulates transcription and translation in ovarian granulosa cells, which are essential for female reproductive endocrine regulation (Dai *et al*., 2021). The timing of fully grown germinal vesicle oocytes to reach the MII stage *in vitro* for different species and the various approaches for improving maturation are presented in Table II (Gordon, 2003; Baruffi *et al*., 2004; Somfai *et al*., 2012). Enrichment of maturation media with cumulus cells, hormones, growth factors, and other factors improves oocyte maturation and embryo development.

## CYTOPLASMIC MATURATION AND NUCLEAR REPROGRAMMING OF RECONSTRUCTED OOCYTES AND DEVELOPMENTAL COMPETENCE

Germinal vesicle nucleus, nucleolar, or cytoplasmic dysfunction of oocytes may be associated with infertility in animal and human oocytes (Fulka *et al*., 2004; Miao *et al*., 2019; Wang *et al*., 2022). Oocyte GV nuclei, nucleoli, MII spindles, and cytoplast transfer techniques have been used for the treatment of oocyte dysfunction due to cytoplasmic, nuclear, or nucleolar reasons or to increase their developmental competence (Fulka *et al*., 2004; Hoseini *et al*., 2016; Benc *et al*., 2018). Hoseini *et al*. (2016) concluded that cytoplast transfer technique is not effective for cytoplasmic maturity of recipient GV oocytes; however, the presence of cumulus cells during oocyte maturation or GV nucleus or nucleolus transfer is effective in increasing the developmental competence of the resulting embryos. Chang *et al*. (2005) found that the developmental incompetency of denuded mouse GV oocytes matured *in vitro* is ooplasmic in nature and is associated with aberrant Oct-4 expression.

One of the fundamental factors for successful assisted reproductive technologies (ART) in reconstructed oocytes is the interaction between the recipient cytoplast and donor nucleus (Polanski *et al*., 2005; Mohammed, 2006; Mohammed *et al*., 2008, 2010; Wani *et al*., 2018). Oocytes at stages GV, ProMI, MI, and MII were enucleated to obtain recipient cytoplasts. The obtained cytoplasts can be used in cell biology for basic research and reprogramming of the introduced germ, embryonic, or somatic nuclei during transfer (Grabarek *et al*., 2004; Polanski *et al*., 2005; Mohammed *et al*., 2008, 2010, 2019). Such approaches might solve the problem of low developmental competence of oocytes from prepubertal or advanced maternal age (Hassold and Chiu, 2003) due to developmental incompetency of ooplasmic or nuclear nature (Bao *et al*., 2003). The following questions arise: Does the transfer of nuclei from non-growing, growing, or aging GV oocytes into fully grown GV cytoplasts solve infertility problem? Does the transfer of MII spindles to competent mature MII cytoplasts rescue infertility in animals and humans? Does the transfer of competent nucleoli from donor fully grown GV oocytes to recipient GV oocytes containing abnormal nucleoli rescues infertility?

**Table II. Duration of oocyte maturation *in vitro* from germinal vesicle to metaphase II stage using different supplements to maturation media in different animal species and human.**

| Species | Duration, h | Treatments | References |
|---|---|---|---|
| Rodents | 17 | Cumulus cells | Grabarek *et al*., 2004 |
| | | Cumulus cells | Mohammed 2006, 2008 |
| | | Cumulus cells | Mohammed *et al*., 2006, 2008, 2010 |
| | | Perfluorooctane | Wei *et al*., 2021 |
| | | CRH and ACTH | Gong *et al*., 2021 |
| Goat | 24 | Cysteamine, leukemia inhibitory factor, and Y27632 | An *et al*., 2018 |
| | | Physiological oocyte maturation | Suresh *et al*., 2021 |
| | | Leptin | de Senna Costa *et al*., 2022 |
| Sheep | 24 | Resveratrol | Zabihi *et al*., 2020 |
| | | Oxygen tension | Sánchez-Ajofrín *et al*., 2020 |
| | | C-type natriuretic peptide | Zhang *et al*., 2018 |
| Cattle | 24 | FF and FCS | Mohammed *et al*., 2005 |
| | | Retinoic acid | Borges *et al*., 2021 |
| | | Lycopene | Residiwati *et al*., 2021 |
| Buffalo | 24 | - | Marin *et al*., 2019 |
| | | Brain-derived neurotrophic factor | Zhao *et al*., 2019 |
| | | Retinoic acid | Gad *et al*., 2018 |
| Rabbit | 24 | Gonadotropin-releasing hormone | Yoshimura *et al*., 1991 |
| | | - | Arias-Álvarez *et al*., 2017 |
| Human | 24 | - | Chian *et al*., 2004 |
| | | - | Baruffi *et al*., 2004 |
| | | Coenzyme Q10 | Ma *et al*., 2020 |
| | | A fertilin-derived peptide | Sallem *et al*., 2022 |
| Camel | 42-48 | - | Wani *et al*., 2018 |
| | | Macromolecule | Moulavi and Hosseini, 2019 |
| | | Roscovitine | Wani and Hong 2018; 2020 |
| Pig | 48 | Dihydroartemisinin | Luo *et al*., 2018 |
| | | Zearalenone | Wang *et al*., 2022 |
| | | CRH and ACTH | Gong *et al*., 2021 |
| | | Allicin | Li *et al*., 2022 |



Therefore, the techniques of enucleation of denuded GV, cumulus-enclosed GV, and enucleolation of GV oocytes were adapted for such studies (Grabarek *et al*., 2004; Mohammed *et al*., 2008, 2010, 2019). Our unique enucleation and enucleolation techniques (Figs. 5 and 6) of denuded and cumulus-enclosed GV oocytes enabled us to confirm the role of cumulus cells, nuclear material, and nucleoli on oocyte maturation and embryo development. Complete enucleation of denuded GV oocytes removes the entire GV nucleus. Therefore, the resulting cytoplast does not contain any GV components or cumulus effects. Selective enucleation of denuded GVs involves the removal of nuclear membrane, leaving the nucleolus and nuclear sap in the cytoplast; however, selective enucleation of cumulus-enclosed GVs leaves the effect of nucleolus, nuclear sap, and cumulus cells in the cytoplast (Fig. 5). Therefore, the aforementioned techniques and enucleolation confirmed the role of cumulus cells, nuclear material, and nucleolus in oocyte maturation and developmental competence.

Our results of selective enucleation of GV oocytes yielded GV cytoplasts in similar quantity as did complete enucleation of GV oocytes (Mohammed, 2006; Mohammed *et al*., 2008, 2010). Therefore, the aforementioned techniques and enucleolation confirmed the role of cumulus cells, nuclear material, and nucleolus in oocyte maturation and further developmental competence of embryos. Our results indicated that GVsel cytoplasts provided proper timing of maturation after reconstruction with GV, contrary to GV cytoplasts, which accelerated timing of maturation. However, this did not improve haploidization, as measured with abnormal nuclear morphology. Abnormal acceleration of maturation cannot be rescued by any particular cell cycle stage or developmental stage of blastomeres 1/2, 1/4

or 1/8 used as nuclear donor. Highest maturation efficiency of GV cytoplasts together with their least disturbance from abnormal meiosis were provided by G2 donor blastomere nuclei (Mohammed, 2006; Mohammed *et al*., 2008, 2010, 2019, 2022).

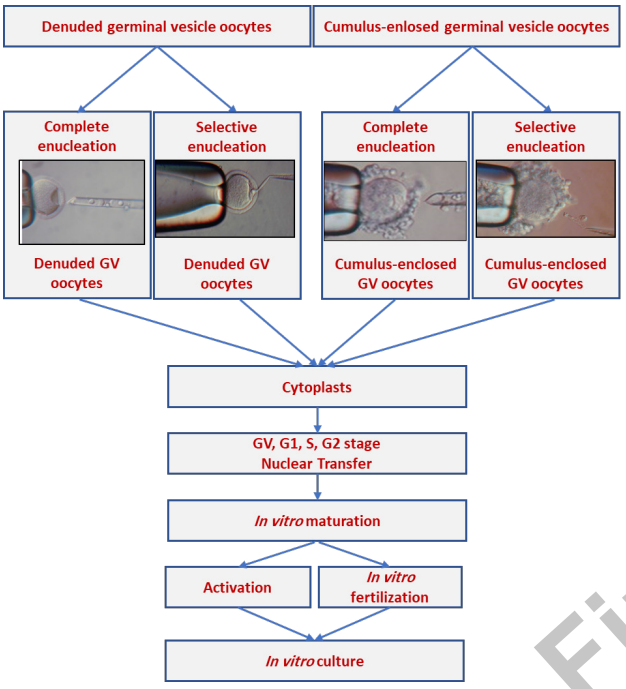


Fig. 5. Obtaining types of recipient germinal vesicle cytoplasts, donor nuclei transfer and further meiotic maturation and developmental competence; GV germinal vesicle nucleus of fully-grown oocyte, G1 gap 1 stage, S S-phase stage, G2 gap 2 stage.

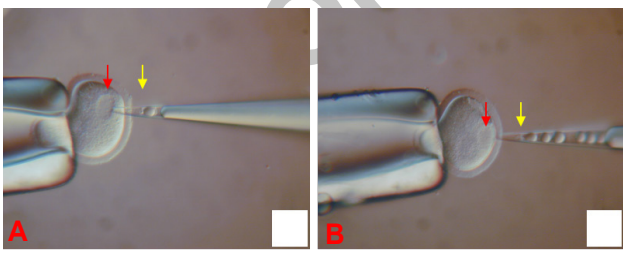


Fig. 6. Enucleolation of fully grown germinal vesicle oocyte (A and B) the nucleolus-free germinal vesicles are visible (red arrows) in the cytoplasm. In the enucleolation pipettes, the nucleolus of GV oocyte undergoing enucleolation (A, yellow arrow) and several nucleoli of previously enucleolated GV oocytes (B yellow arrows) are visible.

The nucleolus and cumulus cells were confirmed to be dispensable for GV oocyte maturation, but the resulting embryos are blocked at the two-cell stage with an anucleolated nucleus (Fig. 7). Therefore, selective enucleation as countered by enucleolation revealed the influence of GV contents on pronuclear formation. Furthermore, complete enucleation of denuded GV oocytes followed by G2/M embryonic or fetal fibroblast nuclear transfer indicate maturation with abnormalities such as acceleration of polar body extrusion, large polar body, and misalignment of chromosomes over the MII spindle, in addition to blocked embryo development at the one-cell stage after activation or fertilization. Surprisingly, no nucleoli were observed in the developed embryos. In addition, upon fertilization, one pronucleus was formed in the cytoplast, and was expected to be somatic, whereas the male pronucleus was not formed and was found as scattered chromosomes in the cytoplast (Fig. 8). This expectation is based on the fact that the pronucleus close to the polar body is somatic and the pronucleus farthest from the polar body is spermatic. This was confirmed through maturation of GV cytoplasts for 17 h followed by fertilization. Fertilizing sperm in the cytoplasm remained in a highly condensed head form (Mohammed *et al*., 2008, 2010, 2019, 2022).

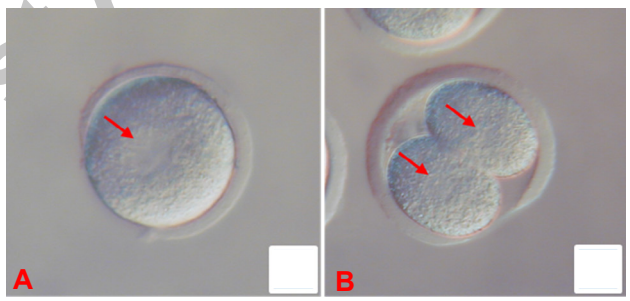


Fig. 7. Pronuclei (arrow) in the zygote (A) and nuclei (arrow) in 2-cell embryo (B) obtained from enucleolated and fertilized fully grown germinal vesicle oocytes. Nucleoli are not visible both in pronuclei and in blastomere nuclei. In the zygote the second pronucleus is out of focus.

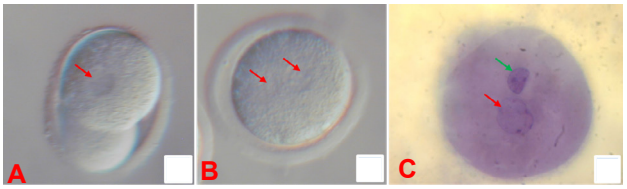


Fig. 8. Pronuclear morphology in GV (A) and GVsel cytoplasts (B and C) reconstructed with embryonic/somatic nuclei and fertilized after *in vitro* maturation (light microscope, DIC). (A) One pronucleus formed of somatic origin without nucleoli (arrow) and extruded large PB; (B) Two pronuclei were formed of somatic and sperm origin and both contained nucleoli; (C) stained two pronuclei were formed of somatic (green arrow) and sperm (red arrow) origin and both contained nucleoli.

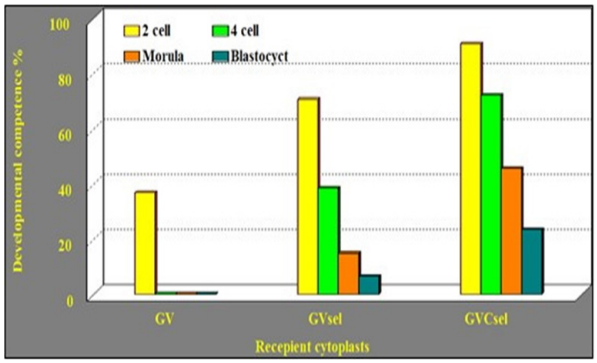


Fig. 9. Effects of nuclear material and cumulus cells on developmental competence of cytoplasts reconstructed with ½-blastomere nuclei. GV removal the whole germinal vesicle of GV oocyte, GVsel removal nuclear material and membrane leaving nucleolus and nuclear sap in the cytoplast of denuded GV oocytes, GVCsel removal nuclear material and membrane leaving nucleolus and nuclear sap in the cytoplast of culumus enclosed GV oocytes.

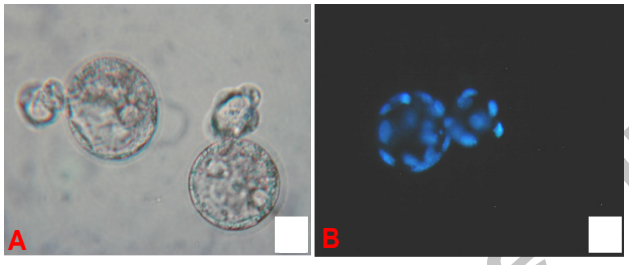


Fig. 10. Hatching blastocysts developed from GVCsel cytoplasts reconstructed with 1/2-blastomere nuclei at G2/M phase (A) two hatching blastocysts under light microscope (DIC) and (B) one hatching blastocyst after Hoechst staining (fluorescence microscope).

Upon selective enucleation of denuded GV oocytes, reconstructed with G2/M embryonic nucleus, followed by maturation and fertilization, both pronuclei were formed in addition to further developmental competence to morula/blastocyst (Fig. 9 and 10). Although activated reconstructed GVsel cytoplasts were also cleaved and few of them developed to the blastocyst stage the frequency of cleavage to the 4-cell stage and blastocyst formation was evidently lower than those of reconstructed GVCsel cytoplasts (38.5% vs 71.4% and 7.7% and 23.8%, respectively). Also, the mean cell number in the obtained blastocysts was slightly higher in blastocysts derived from activated reconstructed GVCsel cytoplasts. What is deserved to observe is the absence or lower cells of inner cell mass in the cloned developed blastocysts over somatic or embryonic nuclear transfer to GVCsel cytoplasts compared to normal developed blastocysts. The number and quality of inner cell mass is the most important character of the developed blastocysts to give birth. Zhu *et al*. (2022) reported that the poor morphology of inner cell mass (grade C) of the developed embryos increases birth weight and large for gestational age compared to inner cell mass of grade A. in addition, Plana-Carmona *et al*. (2022) confirmed that the trophectoderm acts as a niche for the inner cell mass through C/EBPα-regulated IL-6 signaling. Therefore, further studies are required for investigating the reasons of absence or poor quality inner cell mass of the developed blastocysts from GV cytoplasts after embryonic nuclear transfer.

This very interesting technique of enucleation denuded or cumulus-enclosed GV oocytes could be applied with visible GV nucleus as in human, rodent and rabbit oocytes. In ruminant GV oocytes, ultra-centrifugation to 15,000 rpm is required for visualization of GV nucleus due to lipid droplets and the cumulus is stripped off during such centrifugation. In addition, the GV nucleus could be visible approximately in two third of the centrifuged GV oocytes.

Concerning the cell cycle stage of the introduced donor nuclei, our results and others indicate that G0/G1 cells or nuclei could be transferred to metaphase II cytoplasts, whereas G2/M cells or nuclei could be transferred to germinal vesicle cytoplasts (Wilmut *et al*., 1997; Mohammed *et al*., 2008, 2010, 2019, 2022). Donor cells or nuclei introduced in the S phase to the germinal vesicle cytoplast result in abnormalities in oocyte maturation and block further developmental competence of embryos. To the best of our knowledge, embryonic or somatic nuclear transfer to metaphase II cytoplasts results in deliveries with low efficiency (Campbell *et al*., 1996; Wani *et al*., 2018), whereas with embryonic or somatic nuclear transfer to GVCsel cytoplast, the embryonic development reached to the hatched blastocyst stage (Mohammed *et al*., 2008, 2010). Upon morula and blastocysts transferred to the uterine of surrogate mothers, no delivery was confirmed in mice (Mohammed unpublished data).

Our complete and selective enucleation of GV yielded GV cytoplasts with high efficiency (Wilmut *et al*., 1997; Mohammed *et al*., 2008, 2010, 2019, 2022). The sole success of the recipient germinal vesicle cytoplast is the meiotic maturation of germinal vesicle karyoplasts; germ cells; primary or secondary spermatogonia (Ogura *et al*., 1998; Zhang *et al*., 2015). Germinal vesicle recipient cytoplasts are required in basic research for investigations of cell biology and donor cell reprogramming. Further studies are still required for improvement the outcomes of manipulation germinal vesicle oocytes in different animal species and human as well.

## CONCLUSIONS

Oocytes grow and develop during fetal and postnatal periods within several distinct ovarian follicles, and they ovulate in each estrous or menstrual cycle from ovarian follicles after puberty. Trials were unsuccessful when germ cells were used for *in vitro* embryo production during the fetal period; however, they succeeded when germ cells were used of the prepubertal and post pubertal periods for *in vitro* embryo production. The higher the follicle diameter and oocyte quality, the higher the maturation, fertilization, and developmental competence of the embryo. Estrous or menstrual cycle synchronization and superovulation protocols for ovarian follicles have been used in different species for embryo transfer. In addition, ovarian tissue transplantation has been adopted for the treatment of infertility and conservation of species. Furthermore, defects in oocytes can be repaired via the nucleolus, germinal vesicle, and spindle transfer to the recipient oocyte or cytoplast. Primary and secondary spermatocytes and spermatids have been successfully used to fertilize GV and MII oocytes. Furthermore, germinal vesicle, embryonic, and somatic cells at different cell cycle stages (G0, S, and G/M stages) have been transferred to different cytoplasts (GV, ProMI, MI, and MII stages) with variable results. Assisted reproductive techniques (ART) are used to treat infertility, save endangered species, enhance meat and milk production through *in vitro* manipulations of germ cells, follicles, oocytes, and sperm. Studies on the kinetics or progression of oocyte maturation and further developmental competence of embryos are required, in addition to ovarian follicle gene expression. Although assisted reproductive techniques are still relatively rare and expensive, especially in third-world countries, their use has doubled over the past decade and thus necessitates further development.

## DECLARATIONS

*Acknowledgments*

The authors would like to acknowledge the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (GrantA051).

*Funding*

The study was funded by Funding of Scientific Research Deanship of King Faisal University (GrantA051)

*IRB approval*

The approval of the study was granted by the The Ethical Research Committee of King Faisal University.

*Ethical statement*

The proceedures and material of the current study was approved of the Scientific Research Deanship Ethical Standards of King Faisal University.

*Statement of conflict of interest*

The authors have declared no conflict of interest.

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