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# An Insight into the Roles of Zona Pellucida in Growth and Development of Mammalian Ocytes and Embryos: Changes of Age-related and Cryopreservation: A Review

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## ABSTRACT

The zona pellucida (ZP) is a extracellular transparent coat surrounding the oocyte and preimplantation embryo in mammals. The ZP separates between the surrounding cumulus cells and oocyte. It plays a pivotal role in oocyte growth and fertilization and early embryo development. Zona pellucida formation and glycoproteins, roles of ZP on female fertility and infertility, ZP micromanipulation on embryo development and changes of ZP upon vitrification of oocytes is discussed. Zona pellucida is formed of glycoproteins during ovarian follicle growth and development. It protects the oocyte from physical or chemical damage, initiates fertilization, prevents polyspermy and supports early embryonic development. The ZP is responsible for recognition between oocytes and sperm. Mutations in glycoproteins cause female infertility characterized by empty follicle syndrome and oocyte degeneration. The ZP removal is detrimental for embryo implantation. Furthermore, there is a biophysical role of ZP during vitrification of oocytes. Hence, the current review article was designed to collect, discuss and highlight the characters of zona pellucida, roles in female fertility and infertility, ZP micromanipulation on embryo development and changes of ZP upon vitrification.

**Key words:** Aging, Embryos development, Follicles, Gene expression, Oocytes morphology, Zona pellucida.

The term ZP was first described by von Baer in 1827 when describing human oocytes. The zona pellucida is a thick extracellular coat that is synthesized during follicular growth and development (Modliński, 1970, Moros-Nicolás *et al.,* 2021) (Fig 1). Zona pellucida components are presented in fetal ovary before follicle formation (Törmälä *et al.,* 2008). Substantial chnages occurred in zona pellucida formation and thicknes during the stages of oocytes and embryos. Surprinsingly, the features of the ZP of embryos produced *in-vitro* differs significantly from its *in-vivo* counterpart, influencing metabolism and requiring effort to open at hatching (Madani *et al.,* 2022).

It surrounds mammalian oocytes and cleaved embryos till hatched blastocyst stage (Fig 2A-D) (Mohammed *et al.,* 2022). Zona pellucida of human oocytes consists of four glycoproteins whereas that of mouse, rabbit and cattle oocytes consists of three glycoproteins (Moros-Nicolás *et al.,* 2021). Alterations in zona pellucida surface morphology due to age-advancement has been found to prevent spermatozoa binding to oocytes and comporomise fertilization (Ishikawa-Yamauchi *et al.,* 2022).

Zona pellucida is an essential part for oocyte and embryo growth and development in mammalian species (Gordon, 2003; Mohammed, 2006; Mohammed *et al.*, 2022). The zona pellucida is made up of sugars attached to proteins; glycoproteins. These glycoproteins allows small molecules to pass through to the cytoplast of oocytes. In addition, the zona pellucida plays a critical role in protecting the egg, selecting sperm and initiating fertilization (Gupta *et al.,* 2012). In addition, the pivotal role of zona pellucida in vitrification of oocytes has been indicated (Choi *et al.,* 2015). Furthermore, cat embryos generated *in vitro* without zona pellucida are capable of developing but exhibit decreased implantation rate and abnormal gene expression (Veraguas-Davila *et al.,* 2021). Additionally, it has been mentioned in several studies that human oocytes recovered without a zona pellucida were successfully fertilized and established normal pregnancies that led to healthy offspring (Ueno *et al.*, 2014; Hu and Trolice, 2016; Metwalley *et al.*, 2020). Hence, the current review article was designed to collect, consolidate and highlight the characters of zona pellucida, role in female fertility and infertility, ZP micromanipulation on embryo development and changes of ZP upon age-related vitrification of oocytes.

The current review was designed according to the procedure approved by Deanship of Scientific Research, King Faisal University, Saudi Arabia from October to

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February 2024. The transmission and scanning electron or confocal laser scanning microscopes were used for visualization of zona pellucida (Vanroose *et al.,* 2000; Familiari *et al.,* 2006; Choi *et al.,* 2013). In addition, biochemical techniques including isolation and purification of ZP proteins, mass spectrometry and enzyme-linked immunosorbent assay were used for characterization different proteins to elucidate their expression levels and potential roles (Priel *et al.,* 2020; Moros-Nicolás *et al.,* 2021). Besides, biophysical techniques were used to measure the mechanical properties of the ZP allowing for measurements of its mechanical properties and interactions with other biomolecules (Clark, 2010; Wassarman and Litscher, 2021). Finally, applications were used to alleviate the negative effects of aging oocytes in mammalian species (Qu *et al.,* 2023a,b).

The articles of zona pellucida are related to ZP formation and function, roles in fertility and infertility of females, changes of ZP upon age-related and vitrification of oocytes and ZP micromanipulation in oocytes and embryos (Wassarman, 2008; Wassarman and Litscher, 2021; Aljubran *et al.,* 2023).

## Zona pellucida formation

Zona pellucida is molded from the secretions of the surrounding granulosa cells and the oocyte at the early stage of follicular growth (Gook *et al.,* 2008; Litscher and Wassarman, 2020). Zona pellucida first appears when the oocyte begin to grow within the ovarian cortex. Transcription of zona pellucida genes occurs during oocyte growth (Liang and Dean, 1992; Rankin and Dean, 1996). Zona pellucida is encoded by single-copy genes located on different chromosomes (Liang *et al.,* 1990; Liang and Dean, 1992, Epifano *et al.,* 1995). Genes encoding ZP proteins are conserved among mammalian species with distinct domains defined by exon/intron boundaries that contain consensus splice donor/acceptor sites.

The oocyte is surrounded by specialized granulosa cells within the ovary during the early stages of follicular development (Mohammed *et al.,* 2022). These cells communicate with the oocyte through gap junctions and provide essential nutrients and signals for oocyte growth (Gordon, 2003). Genes encoding specific zona pellucida proteins are activated within the growing oocyte (Epifano, 1995; Wassarman, 2008; Wassarman and Litscher, 2021). These proteins, primarily called ZP1, ZP2, ZP3 and ZP4 are unique to oocytes (Gupta 2018, 2021). Newly synthesized zona pellucida proteins are packaged into vesicles and transported to the oocyte's surface. Upon reaching the plasma membrane, they are secreted into the space between the oocyte and the granulosa cells. Zona pellucida proteins outside the oocyte undergo self-assembly, forming long, thread-like structures called fibrils. These fibrils further cross-link, creating a dense meshwork that gradually expands around the oocyte. As follicular development progresses, the zona pellucida thickens and becomes more complex and a highly organized dynamic structure (Gupta, 2018). The thickness ranges from 2 to 20 µm for fully grown germinal vesicle oocytes of different mammalian species, the ZP thickness of human oocyte is about 18 µm and in mouse oocyte it is about 6 µm (Wassarman and Litscher, 2021).

Additional layers and modifications to the ZP proteins occur to fine tune its properties for later functions (Prasad *et al.,* 2000). Upon ovulation, the mature oocyte, surrounded by the zona pellucida, is released from the ovary (Aljubran *et al.,* 2023). During fertilization, sperm must bind to and penetrate the zona pellucida for successful fusion with the oocyte (Gadella, 2010). Specific molecules on the sperm surface interact with ZP proteins, triggering the acrosome reaction to release enzymes for sperm penetration. The zona pellucida undergoes modifications after fertilization to prevent polyspermy (Gupta and Bhandari, 2011; Gupta, 2018; Fahrenkamp *et al.*, 2020; Evans *et al.,* 2020).

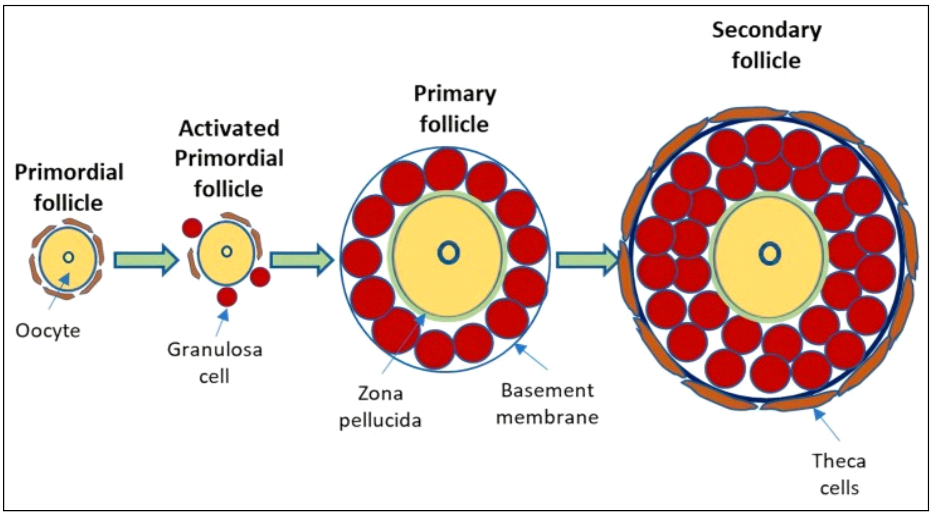


**Fig 1:** Formation of zona pellucida during ovarian follicle formation.

Collectively, the zona pellucida protects the oocytes and early developing embryos from harmful agents in the surrounding environments and provides a supportive structure for proper embryo configuration during its initial stages (Moros-Nicolás *et al.,* 2021). Finally, the full expanded blastocyst eventually hatches out of the zona pellucida before implantation in the uterus (Han *et al.,* 2024).

## Zona pellucida and female fertility

The zona pellucida plays several pivotal roles in female fertility where it acts as a guardian for the oocytes and facilitates fertilization and early embryonic development (Tokuhiro and Dean, 2018). The porous in zona pellucida was involved in the sperm binding process (Familiari *et al.,* 1992). The glycoproteins that form the zona pelluclida contain specific oligosaccharide chain residues that have been related to sperm receptor activity (Tulsiani *et al.,* 1997; Velásquez *et al.,* 2007).

The infertility problem of women has increased in the past dozens of years in the world (Balasch, 2010). Therefore, assisted reproductive techniques have steadily increased for treatment of infertility (Mouzon *et al.,* 2009). Sun *et al.,* (2023) found that novel variants in ZP1, ZP2 and ZP3 was associated with abnormal zona pellucida and empty follicle syndrome. For investigating mouse ZP genes and female fertility, gene targeting was used to establish mouse lines in which mZP genes were inactivated (Wassarman and Litscher, 2021). Female mice that are homozygous nulls for either *mZP2* (*mZP2-/-*) or *mZP3* (*mZP3-/-*) produce oocytes that lack a zona pellucida and these females are completely infertile (Rankin *et al.,* 1996, 2001; Liu *et al.,* 1996, 2023; Liu *et al.,* 2023).

## Zona pellucida micromanipulation

Zona pellucida formations and functions are played pivotal roles in assisted reproductive techniques including *in vitro* fertilization (Malter and Cohen, 1989; Tanihara *et al.,* 2013). The techniques used include removal or thinning or opening the zona pellucida either by chemically, mechanically, or a laser beam to facilitate sperm binding and fertilization in cases of infertility (Laufer, 1991; Cohen *et al*., 1992; Gordon 2003; Alteri *et al*., 2018; Al Jubran *et al*., 2023) (Fig 3A-C).

The empty zona pellucida can be used as a surrogate zona pellucida for a single embryo blastomere or the split embryos (Gordon, 2003; Tang *et al.,* 2012). The empty zona pellucida can be created mechanically by removing and discarding the embryonic blastomeres (Tang *et al.,* 2012). In addition, the empty zona pellucida can be found during oocytes retrieval, which might be attributed either to mechanical factors by applying high suction pressure or due to the degeneration of the cytoplast through the process of apoptosis (Zhou *et al.,* 2019; Siristatidis *et al.,* 2021). Additionally, the cracked empty zona pellucida can be obtained after hatched blastocyst (Mohammed *et al.,* 2010; 2019). The empty and opened ZP might be used for the aggregation of multiple embryos to form chimeras (Mintz, 1962). These techniques were gradually abandoned due to the minimal benefits compared to the major side effects as

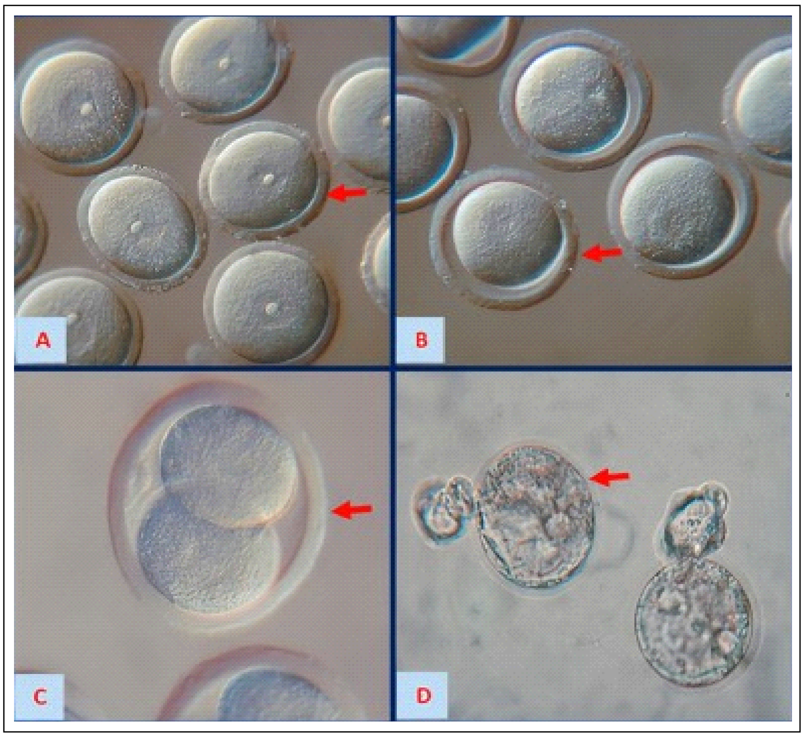


**Fig 2:** Mouse zona pellucida (red arrow) surrounding the GV oocytes (A), Prometaphase I oocyte (B), cleaved embryos (C) and hatching blastocysts (D).

direct trauma to the embryo, the suboptimal environment, increased the risk of twin pregnancies (Datta *et al.*, 2015; Amstrong *et al.*, 2019; Madani *et al.,* 2022).

## Changes of zona pellucida in aged-oocytes

The ZP undergoes several age-related alterations that can negatively impact fertilization and embryo development (Ishikawa-Yamauchi *et al.,* 2022). Changes in thickness, density and glycosylation patterns has been suggested. Several studies suggest a trend of decreasing zona pellucida thickness with age in some species leading to be more susceptible to damage (Takahashi *et al.,* 2011). Such age-related changes in the zona pellucida may impair sperm binding with ZP and preventing fertilization. Age-related alterations in the surface morphology of ZP prevent sperm binding to oocyte and affect fertilization has been confirmed (Ishikawa-Yamauchi *et al.,* 2022). The last study suggested that the decrease in fertilization ability of mouse aged-oocytes is not due to gene expression in MII oocytes, but to inhibition of sperm binding to surface of the zona pellucida.

Besides, zona pellucida may become stiffer with age making the developing blastocyst not hatching, which potentially hindering implantation. Lastly, reduced fertilization and pregnancy rates are the expected of aged-oocytes (Bresnahan *et al.,* 2022) in addition to increased risk of aneuploidy due to sperm carrying abnormal chromosome numbers. Therefore, the crucial strategies to improve fertility in aged-oocytes of mammalian species might require ZP modification or assisted hatching techniques in aged-oocytes due to decrease sperm binding to oocytes. In addition, the impact of environmental factors and lifestyle choices on ZP quality might open avenues for preventative measures to preserve fertility potential. Researchers by studying the aged-oocytes changes in the zona pellucida can gain invaluable insights into female fertility decline and develop potential strategies to improve reproductive outcomes for women and superior mammalian species as well.

## The applications used to ameliorate the negative effects of aged-oocytes

Oocyte aging occurs in preovulatory and postovulatory oocytes (Tarin *et al.,* 2000). Preovulatory oocyte aging is occurred due to ovarian aging in reproductive aged women over 35 years old or ovulation does not occur in time (Broekmans *et al.,* 2009) and also it is occured in experimental and domestic animals (Tarin *et al.,* 2000). The postovulatory oocyte aging occurs if fertilization does not occur during an optimal period after ovulation or in *in vitro* culture (Tarin *et al.,* 2000; Takahashi *et al.,* 2011; Nicholas *et al.,* 2023). Advanced reproductive age in women is generally referred to as over 35 years (Heffner, 2004). The advanced age of females was associated decline fertility because of oocyte aging (Tarin *et al.,* 2002). Postovulatory

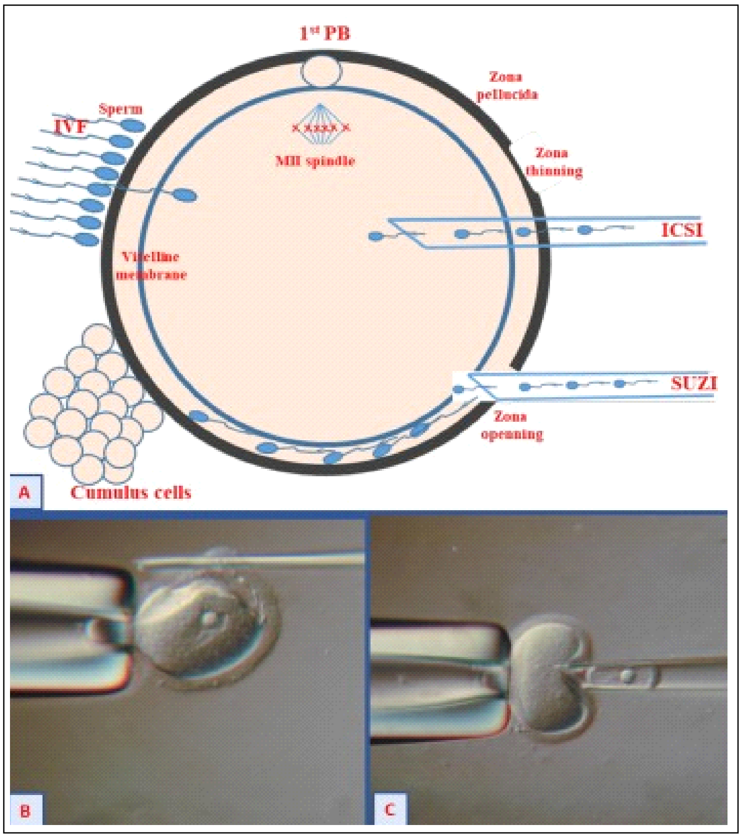


**Fig 3:** Zona pellucida micromanipulation for assisted fertilization (A) and during oocytes enucleation (B and C).

oocyte aging either *in vivo* or *in vitro* mostly results in low fertilization rate, polyspermy and abnormal embryo development. These abnormalities resulted in decreased litter size in animals, low pregnancy rate and increased spontaneous miscarriage in humans (Huhtinen *et al.,* 1996; Wilcox *et al.,* 1998) (Fig 4).

There are different reasons for the negative effects over the oocyte aging. Zhang *et al.,* (2013) found that goat oocytes started to age at 30 hours *in vitro* culture and gene expression patterns changed of aged-oocytes. Babayev *et al.,* (2016) concluded that aging-oocytes is associated with changes in mitochondrial dynamics, function and mtDNA quantity. The close relationship between aging-oocyte and telomere shortening has been found (Ozturk, 2024).

Applications were explored to ameliorate the negative effect of aging-oocytes of human and animals as well. Autologous platelets mitochondrial microinjection was found to improve fertility rate in aged oocytes (Li *et al.,* 2010). Yao *et al.* (2024) found that Apigenin delays postovulatory oocyte aging by reducing oxidative stress through SIRT1 upregulation. In addition, melatonin treatment was found to ameliorate histone modification disorders in mammalian aged oocytes by neutralizing the alkylation of HDAC1 (He *et al.,* 2023). Furthermore, melatonin also was found to restore the declining maturation quality and early embryonic development of oocytes in aged mouse oocytes (Qu *et al.,* 2023a). Kaempferol has been found to ameliorate aging of *in vivo* and *in vitro* mouse postovulatory aged-oocyte (Zeng *et al.,* 2022). Anti-ovarian aging herbal formulation was found promising to promote oocyte *in vitro* maturation of oocytes from advanced age mice (Yang *et al.,* 2024). Besides, near-infra red fluorophore IR-61 improves oocyte quality in aged mice *via* mitochondrial protection (Qu *et al.,* 2023b). Collectively, in our point of view, all the treatments to alleviate the negative effects of aging in oocytes are helpful to a degree for improving oocyte maturation, fertilization, preimplantation and post implantation embryonic development.

## Effects of differences between zona pellucida *in vitro* or *in vivo* produced

The features of the zona pellucida of *in-vitro* produced embryos differs significantly from its *in-vivo* counterpart. Zona pellucida hardening of embryo culture *in vitro* is indicated (Cohen, 1991) leading to problems of early embryo development and hatching of expanded blastocyst. The embryos *in vivo* produced are showing a continuous increase in elasticity in addition to a decrease in the resistance to proteolytic digestion and various morphological alterations during development due to their development in a dynamic environment including fallopian and uterus fluids (Roth *et al.*, 1994). Hardening ZP may hamper the communication of embryos with the environment, which deceases the access to growth factors and other ligands (Brown *et al.*, 1990). Additionally, A hardened ZP may decrease the number and size of cytoplasmic projections, which pass through the pores of the zona pellucida and consequently leads to implantation failures (Vajta *et al.,* 2010). Besides, zona hardening may delay hatching, which may exhaust the embryo in the crucial developmental stage of early differentiation (Gonzales and Bavister, 1995). de Almeida Ferreira Braga *et al.* (2010) investigated the zona pellucida birefringence (ZPB) in *in vitro* and *in vivo* matured oocytes. They concluded that zona pellucida birefringence may be a useful tool to predict embryo quality for metaphase-II oocytes. In addition, Held *et al.* (2012) found that ZPB correlates with developmental competence of bovine oocytes classified by cumulus-enclosed oocyte morphology, G6PDH activity and maturational environment.

### Changes of zona pellucida upon vitrification

Vitrification, a rapid freezing technique, used for cryopreservation of oocytes and embryos may induce changes in the zona pellucida that can negatively impact their developmental competence and hatching. Firstly, the presence of zona pellucida improved the post vitrification survival of oocytes compared to those oocytes without zona

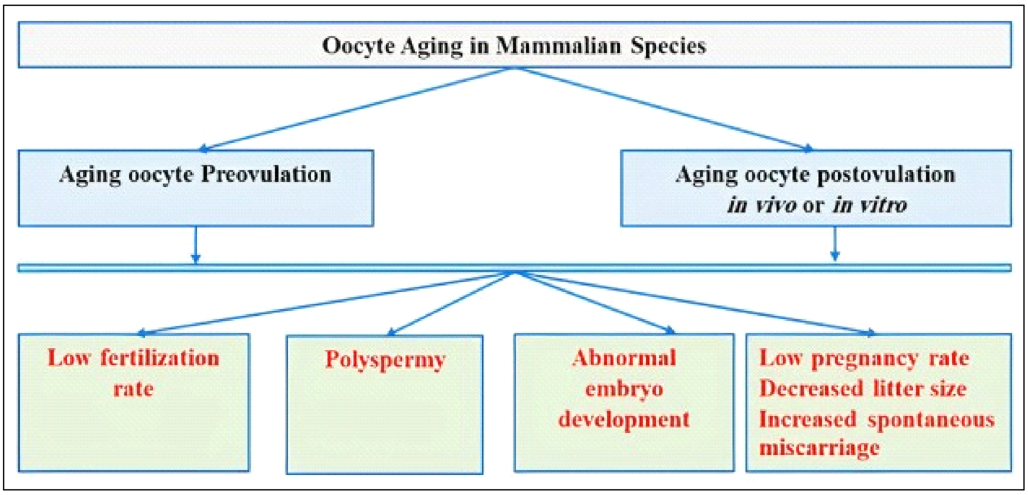


**Fig 4:** Negative effects of oocyte aging in mammalian species.

pellucida (Choi *et al.,* 2015). Secondly, the changes that may occur to the zona pellucida upon vitrification include hardening, thinning and Cracking (Manna *et al.,* 2001; Moreira da Silva and Metelo 2005; Wiesak *et al.,* 2017). These changes in the zona pellucida over vitrification led to reduced fertilization rates, impaired embryo development and increased cell death (Choi *et al.,* 2015). Therefore, adapting a new assisted reproductive techniques that may keep the characters of ZP and their receptors for sperm binding in addition to assisting of embryo hatching are required.

## CONCLUSION

The zona pellucida is a vital structure formed during ovarian follicle growth and development and surrounding the oocytes and preimplantation embryos till hatching process. It protects and supports the oocytes and embryos during growth, fertilization and early embryo development. Exploring zona pellucida changes in aged-oocytes and *in vitro* culture systems may still provide invaluable insights into fertility and potential advancements in assisted reproductive techniques for developing better fertility treatments.

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### Conflict of interest

There is no conflict of interest for authors to declare.

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