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Melatonin improves the effect of cryopreservation on human oocytes by suppressing oxidative stress and maintaining the permeability of the oolemma

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Running title: Melatonin protects cryopreserved human oocytes

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Abstract

Cryopreservation causes cryoinjury to oocytes and impairs their developmental competence. Melatonin (MLT) can improve the effect of cryopreservation in animal oocytes. However, no such studies on human oocytes have been reported. In this study, collected *in vitro*-matured human oocytes were randomly divided into the following groups: fresh group, MLT-treated cryopreservation (MC) group, and no-MLT-treated cryopreservation (NC) group. After

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vitrification and warming, viable oocytes from these three groups were assessed for their mitochondrial function, ultrastructure, permeability of oolemma, early apoptosis, developmental competence, and cryotolerance-related gene expression. First, fluorescence staining results revealed that oocytes from the 10^{-9} M subgroup showed the lowest intracellular reactive oxygen species and Ca^{2+} levels and highest mitochondrial membrane potential among the MC subgroups (10^{-11}, 10^{-9}, 10^{-7}, and 10^{-5} M). In subsequent experiments, oocytes from the 10^{-9} M-MC group were observed to maintain the normal ultrastructural features and the permeability of the oolemma. Compared with those of the oocytes in the NC group, the early apoptosis rate significantly decreased ($P<0.01$), whereas both the high-quality cleavage embryo and blastocyst rates significantly increased (both $P<0.05$) in the oocytes of the 10^{-9} M-MC group. Finally, single-cell RNA sequencing and immunofluorescence results revealed that aquaporin (*AQP*) *1/2/11* gene expression and AQP1 protein expression were upregulated in the MC group. Therefore, these results suggest that MLT can improve the effect of cryopreservation on human oocytes by suppressing oxidative stress and maintaining the permeability of the oolemma.

Introduction

Melatonin (N-acetyl-5-methoxytryptamine, MLT), a derivative of tryptophan, is produced not only in the pineal gland but also in neuronal cells[1], skin[2], and oocytes[3], and is a potent free radical scavenger and natural antioxidant[4]. Previous studies have demonstrated that MLT and its metabolites act as powerful direct scavengers of free radicals and indirect antioxidants[4] by regulating the gene expression of antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase[5]. Recently, MLT has been widely used as an effective and safe cytoprotective agent in assisted reproductive technology (ART). Accumulating evidences have indicated that the addition of MLT in the culture medium can effectively improve gamete and embryo quality[6-8].

Oocyte cryopreservation is an important technique in human ART, and it has great application value in modern medicine. The applications of oocyte cryopreservation include preserving female

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fertility for women suffering from cancer and reduced ovarian function as well as postponing childbearing, preventing hyperstimulation syndrome, and oocyte banking for donation[9]. In recent years, although oocyte cryopreservation technology has been optimized and has achieved a high survival rate after cryopreservation, the blastocyst rate and the clinical pregnancy rate of cryopreserved oocytes are still lower than those of the fresh oocytes. Recent studies have shown that the blastocyst rate is 3.5–21.4%[10-12] and the clinical pregnancy rate is 10.7%[13] of *in vitro*-matured metaphase II (IVM-MII) human oocytes after cryopreservation. Therefore, it is crucial to improve the quality of vitrified–warmed oocytes.

Vitrification is an ultrafast cooling method that uses high concentrations of cryoprotectants for dehydration and avoids the formation of ice crystals during oocyte cryopreservation[14]. However, this technique still causes damage to the oolemma, probably due to the toxicity of cryoprotective agents (CPAs) and high osmotic shock[15]. To date, studies concerning oocyte cryopreservation have mainly focused on the comparison of freezing methods and the choice of cryoprotectant type, concentration, and exposure time, such as increasing the total solute concentration with different cryoprotectants, increasing the degree of cellular dehydration, and changing the speed of cooling and/or warming[16]. However, improvements in these cryopreservation technologies have not yielded satisfactory results. It has been demonstrated that increased oxidative stress may be responsible for reducing the quality of vitrified–warmed oocytes[17]. One of the major sources of oxidative stress is the accumulation of reactive oxygen species (ROS)[18], which may be a crucial mediator of damage to proteins, DNA, lipids and the cell membrane[19], thereby disrupting oocyte structure and function. Recently, it was reported that supplementing vitrification medium with 10^{-9} M MLT could increase the quality of vitrified–warmed bovine[20] and mouse oocytes[21], and improve their subsequent development by reducing intracellular ROS. In addition, some reports on the ameliorative effects of MLT in protecting sperm from oxidative stress during cryopreservation have also emerged, including reports in mice[22], rams[23], boars[24], stallions[25], bulls[26], and humans[27-29]. However, to date, no research has used MLT as a cryoprotectant additive for the cryopreservation of human oocytes, and limited information is available about the mechanism of action of MLT in protecting cryopreserved human oocytes from cryoinjury. This may be related to

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the fact that human oocytes are a precious and scarce resource. In our previous study, we successfully used MLT for *in vitro* maturation (IVM) culture of discarded immature human oocytes from the controlled ovarian hyperstimulation (COH) cycle. We obtained a large number of IVM-MII human oocytes with high development potential[30], thereby solving the problem of insufficient research materials available for the cryopreservation of human oocytes.

In the present study, IVM-MII human oocytes with normal morphology derived from the COH cycle were collected and cryopreserved using vitrification–warming media supplemented with MLT, with the aim to systematically explore the effect and mechanism of MLT during the cryopreservation of human oocytes.

Materials and methods

Chemicals and reagents

Unless otherwise specified, all reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Additional details are provided in the Supplementary Materials and Methods under "Chemicals and reagents" subsection.

Ethics statement

This study was conducted at the First Affiliated Hospital of Anhui Medical University Reproductive Medicine Center and was approved by the Committee of Medical Ethics. Informed consent was obtained from all patients participating in this study.

Immature oocyte collection and IVM

Immature human oocytes, including germinal vesicles or metaphase I oocytes, with normal morphology were collected from 236 infertile women (under 35 years of age), followed by IVM culture for 24 h using the method described in our previous report[30]. High-quality IVM-MII oocytes were selected as experimental subjects for this study. Additional details are provided in the Supplementary Materials and Methods under "Immature oocyte collection" and "Protocol for *in vitro* maturation (IVM)" subsection.

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Study design

This study involved three experiments (experiments I, II, and III), in which high-quality IVM-MII human oocytes were randomly divided into a fresh group (F group, as a control, n=154), a no-MLT-treated cryopreservation group (NC group, n=152), and an MLT-treated cryopreservation group (MC group, n=221).

In experiment I, the MC group was further divided into four independent subgroups, and the collected oocytes in each group were subjected to cryopreservation using vitrification–warming media supplemented with different concentrations (10^{-11}, 10^{-9}, 10^{-7}, and 10^{-5} M) of MLT, whereas in the NC group, the oocytes were cryopreserved using media without MLT. Moreover, a group of fresh oocytes was retained as a control. After cryopreservation, mitochondrial function was assessed via fluorescence staining to detect mitochondrial membrane potential (MMP), intracellular ROS and Ca^{2+} levels in oocytes. Ultimately, MLT treatment protected mitochondrial function during cryopreservation, and 10^{-9} M was found to be the optimal concentration. Hence, 10^{-9} M MLT was used for further research (defined as the 10^{-9} M-MC group). In experiment II, to systematically investigate the ameliorative effect of MLT on the cryopreservation of human oocytes, the ultrastructure, permeability of oolemma, early apoptosis, and developmental competence of vitrified–warmed oocytes from the cryopreservation groups (NC group and 10^{-9} M-MC group) and fresh oocytes from the control group were evaluated using transmission electron microscopy (TEM), membrane permeability assays, fluorescence staining, intracytoplasmic single sperm injection (ICSI) insemination, and *in vitro* culturing of embryos. In experiment III, to further analyze the mechanism of MLT in improving the effect of cryopreservation on human oocytes, single-cell RNA (sc-RNA) sequencing and immunofluorescence were used to identify cryotolerance-related genes and detect their protein expression in oocytes collected from the aforementioned three groups. Each experiment was repeated at least three times. The experimental flow chart is shown in Figure 1.

Oocyte vitrification and warming

The vitrification–warming procedure for IVM-MII human oocytes was performed as described

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in our previous study[31]. After vitrification and warming, morphological evaluation of each oocyte was performed under an optical microscope (IX-71, Olympus, Japan). Darkened or degenerated oocytes or oocytes that had lost their original shape were considered as nonviable and only viable oocytes were used in further experiments[32]. Additional details are provided in the Supplementary Materials and Methods under "Preparation of the vitrification and warming media" and "Oocyte vitrification and warming" subsection.

Assessment of mitochondrial function

Indicators of mitochondrial function were detected using fluorescence staining of both vitrified–warmed and fresh oocytes. Intracellular ROS and Ca^{2+} levels and MMP were measured as described in our previous study[30]. Additional details are provided in the Supplementary Materials and Methods under "Quantification of MMP, and intracellular ROS and Ca^{2+} levels" subsection.

TEM evaluation

Ultrastructure of 9 oocytes, from the three groups [F group (n=3), NC group (n=3), and 10^{-9} M-MC group (n=3)], donated by 3 young patients, were evaluated using TEM (FEI Tecnai 10, America). Additional details are provided in the Supplementary Materials and Methods under "Transmission electron microscopy (TEM) protocols" subsection.

Assessment of the permeability of oolemma

Both vitrified–warmed and fresh oocytes were used to detect the permeability of the oolemma. Additional details are provided in the Supplementary Materials and Methods under "Assessment of the permeability of oolemma" subsection.

Assessment of early apoptosis

Early apoptosis in 67 oocytes, from the three groups [F group (n=19), NC group (n=23), and 10^{-9} M-MC group (n=25)] donated by 34 young patients, were measured using an Annexin-V staining kit (Beyotime Biotechnology Inc., Shang Hai, China) according to the manufacturer's instructions. Additional details are provided in the Supplementary Materials and Methods under

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“Assessment of early apoptosis in oocytes” subsection.

Developmental competence

Collected IVM-MII human oocytes were randomly divided into the aforementioned three groups. After vitrification and warming, viable oocytes from the NC group, the 10^{-9} M-MC group, and fresh oocytes from the control group were subjected to ICSI insemination with donated sperm, followed by culturing in a Time-Lapse incubator (Version D; Vitrolife, Sweden) for 6 d. Additional details are provided in the Supplementary Materials and Methods under “ICSI and embryo culture protocols” subsection.

Sc-RNA sequencing

Overall 45 IVM-MII human oocytes were collected from 15 young patients, and each patient’s oocytes were equally divided into three groups [F group (n=15), NC group (n=15), and 10^{-9} M-MC group (n=15)] of 9 samples. Both fresh and vitrified–warmed oocytes were then prepared for sc-RNA sequencing analysis. cDNA library construction and RNA-Seq analysis were performed using a BGISEQ-500 system provided by Beijing Genomics Institution, China. Additional details are provided in the Supplementary Materials and Methods under “Single-cell RNA sequencing analysis” subsection.

Immunofluorescence

Immunofluorescence was performed to examine the protein expression levels of aquaporin 1 (AQP1), AQP2, and AQP11 in fresh and vitrified–warmed oocytes using confocal fluorescence microscopy staining technology. Primary antibodies specific for human AQPs (Abcam, Inc., Cambridge, MA, USA) and anti-rabbit IgG (BioLegend, Inc., San Diego, USA) were used according to the manufacturer’s instructions and as previously reported[33]. Additional details are provided in the Supplementary Materials and Methods under “Immunofluorescence detection of cryotolerance-related proteins” subsection.

Statistical analysis

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Categorical data from the developmental competence analysis were expressed as counts and percentages, and were analyzed using the chi-square test. The remaining data were expressed as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Bonferroni test were used to evaluate the statistical significance of differences between more than two groups. SPSS16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis and P-values <0.05 were considered statistically significant.

Results

Morphology of vitrified–warmed human oocytes treated with different concentrations of MLT

Overall, 406 human oocytes were cryopreserved, and after vitrification and warming, 91.87% (373/406) of them were assessed as viable based on morphological evaluation performed under an optical microscope.

As shown in Figure 2, 10^{-9} M MLT treatment maintained the regular morphology of oocytes, with a uniform cytoplasm and no vacuoles, which was similar to the morphology of fresh oocytes. In contrast, oocytes from the NC group and the 10^{-5} M subgroup displayed abnormal morphology with the presence of intracytoplasmic granulation clusters, vacuolization, or a large perivitelline space. In addition, a slightly rough cytoplasm was observed in oocytes from the 10^{-7} M and 10^{-11} M subgroups. Therefore, we found that the 10^{-9} M MLT treatment protected the morphology of human oocytes during cryopreservation.

Mitochondrial function of vitrified–warmed human oocytes treated with different concentrations **of** *MLT*

It has been reported that the impairment of oocyte quality is closely associated with alterations in mitochondrial function. MLT treatment has been shown to protect the mitochondrial function of mouse oocytes during cryopreservation effectively[34]. Therefore, we detected intracellular ROS and Ca^{2+} levels and MMP to determine the optimal concentration of MLT to protect the quality of vitrified–warmed human oocytes.

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The images, as shown in Figures 3A and 3B, clearly showed that compared with other cryopreservation treatments, the 10^{-9} M MLT treatment decreased ROS generation in human oocytes, and the ROS level was comparable to that of the F group. Interestingly, the data showed that the Ca^{2+} levels in the oocytes of the 10^{-9} M subgroup were significantly lower than that of the other MC groups (all $P<0.05$) (Figures 3C and 3E). The Ca^{2+} level of the F group was comparable to that of the 10^{-9} M subgroup (Figure 3E). Finally, as shown in Figure 3D, compared with the other cryopreservation groups, the 10^{-9} M MLT treatment showed a positive effect by maintaining high MMP levels in vitrified–warmed oocytes (Figure 3F). However, the MMP level of the F group was comparable to that of the 10^{-9} M subgroup (Figure 3F).

Based on the above results, it can be concluded that 10^{-9} M is the optimal concentration of MLT for protecting the mitochondrial function of human oocytes during cryopreservation.

MLT protected the ultrastructure of human oocytes during cryopreservation

Three pairs of human oocytes from 3 patients were used for TEM to evaluate the protective effects of MLT on the ultrastructure of oocytes during cryopreservation. As shown in Figure 4, fresh oocytes were characterized by the abundance of cortical granules (CGs) arranged in single or multiple rows (Figures 4A and 4D), the presence of numerous spherical or elliptical mitochondria with obvious cristae in their matrix (Figures 4B and 4C), and a large number of long, thin, and intact microvilli projecting into the perivitelline space (Figure 4D). However, the vitrified–warmed oocytes in the NC group showed apparent changes in their ultrastructure, such as the number of CGs remarkably reduced (Figures 4A and 4D), most of the microvilli degenerated (Figure 4D), and the mitochondria became obscured and their cristae disappeared. As an indication of more severe damage, vacuolization was also observed in the cytoplasm of vitrified-warmed oocytes in the NC group (Figures 4B and 4C). Interestingly, in the 10^{-9} M-MC group, although the number of CGs in oocytes decreased slightly (Figures 4A and 4D) and the size of the mitochondria shrank slightly, the mitochondrial morphology was clear, and cristae in the matrix were observed (Figures 4B and 4C). These results strongly indicate that MLT treatment effectively protects the cellular ultrastructure from cryoinjury during cryopreservation.

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MLT maintained the water permeability of the oolemma during cryopreservation

For successful cryopreservation, the oolemma structure must be maintained intact and functional[35,36]. The composition and permeability of the membrane directly affect the flux rates of water and CPA[37-39]. Figure 5A shows typical volume changes before (initial volume V_0) and after the hyperosmotic shock, when oocytes attained their minimum volume (Vmin, approximately 36s), and when they reached their new final equilibrium volume (approximately 600s). A significant difference in the hydraulic conductivity of oocytes between the NC group and the other two groups was observed (both $P<0.05$) (Figure 5B), whereas no difference in the ethylene glycol (EG) permeability was observed among the 3 groups (Figure 5C). As reported, a sufficient efflux of intracellular water is necessary to prevent ice formation during cryopreservation[40]. Therefore, in this study, the change of oocyte volume with time during the shrinkage process in 10% EG/M199 medium was further analyzed (Figure 5D). As shown in Figure 5E, the minimum value of the normalized cell volume (V/V_0 min) of oocytes from the 10^{-9} M-MC group was lower than that of the oocytes from the NC group ($P<0.05$) and comparable to that of the F group, which indicated that the intracellular free water of the oocytes in the NC group was not completely depleted during the dehydration process as compared with that of the other groups. Thus, MLT may maintain the water permeability of the oolemma during cryopreservation.

MLT inhibited the aggravation of early apoptosis in human oocytes during cryopreservation

In animal experiments, MLT treatment has been shown to inhibit the aggravation of apoptosis in oocytes during cryopreservation, thereby effectively protecting oocytes' developmental competence[20]. As shown in Figure 6A, the green fluorescence signal of Annexin-V was visible only at the zona pellucida of the oocytes from the F group and the 10^{-9} M-MC group, however substantially stronger signal intensities were observed in the membrane and zona pellucida of the oocytes from the NC group. The early apoptosis rate of oocytes from the 10^{-9} M-MC group (19.58±4.58%, n=25) was significantly lower than that of the oocytes from the NC group (56.55±6.27%, n=23) ($P<0.01$) and comparable to that of the oocytes from the F group (10.32±9.02%, n=19) (Figure 6B). These results indicate that cryopreservation aggravated the

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degree of early apoptosis in human oocytes, but MLT treatment effectively inhibited this exacerbation during cryopreservation.

MLT protected the developmental competence of human oocytes during cryopreservation

Overall 156 IVM-MII oocytes collected from 72 young infertile women (<35 years) who received ICSI were randomly assigned to the following three groups: the NC group (n=55), the 10^{-9} M-MC group (n=51), and the F group (n=50). There was no significant difference in the baseline data of the patients among these groups (Table S1). After vitrification and warming, 96 oocytes survived and were subjected to ICSI, followed by early embryo culture. Figure 6C shows representative developmental images of fresh and vitrified–warmed oocytes after ICSI, including two pronucleus (2PN) and embryos at the 4-cell, 8-cell, and blastocyst stages. The oocytes from the NC group required a longer time from ICSI to blastocyst formation than that of the oocytes from the other two groups, which provided evidence of delayed development due to cryopreservation. Video S1 shows the developmental process of a vitrified–warmed oocyte from the 10^{-9} M-MC group. As shown in Table 1, the fertilization, high-quality cleavage embryo and blastocyst rates in the 10^{-9} M-MC group were significantly higher than those in the NC group [89.58% (43/48) vs. 62.50% (30/48); 65.85% (27/41) vs. 25.93% (7/27); 39.02% (16/41) vs 11.11% (3/27), respectively; all $P<0.05$] and similar to those in the F group [89.58% (43/48) vs. 90.00% (45/50); 65.85% (27/41) vs 67.44% (29/43); 39.02% (16/41) vs. 53.49% (23/43), respectively; all $P>0.05$]. These results indicate that MLT treatment can effectively protect the developmental competence of human oocytes during cryopreservation.

Global gene expression characteristics of MLT-treated human oocytes during cryopreservation

To further analyze the mechanism of MLT in improving the effect of cryopreservation in human oocytes, sc-RNA sequencing was used to analyze the differences in gene expression. Cluster analysis of gene expression levels [fragments per kilobase million (FPKM)] in each sample showed differences in the profiles, which revealed that the RNA sequencing data of this study met the conditions for differential expression analysis. Significantly differentially expressed genes (DEGs) were defined as genes with FPKM>1 and $P<0.05$. In these three groups, we found 4696,

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1362, and 4594 DEGs, of which 875 genes were upregulated and 487 genes were downregulated between the NC group and the 10^{-9} M-MC group; 3740 genes were upregulated and 956 genes were downregulated between the NC group and the F group; and 913 genes were upregulated and 3681 genes were downregulated between the F group and the 10^{-9} M-MC group (Figure 7A).

Interestingly, among these DEGs, we screened for genes related to cryotolerance and found a class of AQPs (Figure 7B), in which the *AQP1/2/11* genes were upregulated in the 10^{-9} M-MC group or the F group as compared to in the NC group (all $P<0.001$) (Figures 7B and 7C, Table S2). In addition, we found that *AQP2* gene was upregulated, whereas *AQP11* gene was downregulated in the 10^{-9} M-MC group as compared to in the F group ($P<0.05$ and $P<0.001$, respectively) (Figures 7B and 7C, Table S2). Eight common Gene Ontology terms indicated that the *AQP1*, *AQP2*, and *AQP11* genes were annotated as integral components of the membrane and were related to water and glycerol transfer (Figures 7D, 7E, and 7F and Table S3). This result is consistent with the previous study showing that AQPs mainly mediate the exchange of water and osmotic CPAs to maintain the permeability of the oolemma during vitrification and warming[33].

MLT treatment could prevent the inhibitory effect of the vitrification procedure on AQP1 protein expression

Finally, the protein expression of AQP1, AQP2, and AQP11 in human oocytes during cryopreservation was detected. For AQP1, AQP2, and AQP11 immunofluorescence staining, 117 IVM-MII human oocytes were collected and randomly divided into the NC group (n=11, n=12, and n=14, respectively), the 10^{-9} M-MC group (n=16, n=11, and n=14, respectively), and the F group (n=14, n=11 and n=14, respectively). As shown in Figures 8A, 8B, and 8C, we found that cryopreservation downregulated AQP1 protein expression in oocytes (Figure 8D, $P<0.01$), but did not downregulate AQP2 and AQP11 protein expression in human oocytes (Figures 8E and 8F). Contrastingly, MLT treatment reversed the downregulation of AQP1 expression induced by cryopreservation (Figure 8D, $P<0.01$), but did not change the expression pattern of AQP2 and AQP11 (Figures 8E and 8F). These results indicate that MLT treatment could prevent the inhibitory effect of the vitrification procedure on AQP1 protein expression.

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Discussion

In recent years, evidence has suggested that MLT acts as an antioxidant to improve the developmental competence of cryopreserved animal oocytes[21]. Therefore, in the present study, we first identified the optimal concentration of MLT for improving the developmental competence of cryopreserved human oocytes.

In this study, experiment I was performed to explore whether MLT could play a positive role in the cryopreservation of human oocytes. Human oocytes are vulnerable to cold shock during cryopreservation[41]. Cryopreservation of oocytes induces the generation of excessive ROS in the oocytes, which aggravates oxidative stress and compromises the quality of oocytes[34]. Our results showed that compared with those from the other cryopreservation groups, the vitrified–warmed oocytes from the 10^{-9} M MLT subgroup had the lowest intracellular ROS and Ca^{2+} levels and the highest MMP. These results indicate that MLT at the optimal concentration (10^{-9} M), can effectively protect the mitochondrial function of cryopreserved oocytes from oxidative stress by reducing ROS levels.

In experiment II, we systematically explored the ameliorative effect of MLT on the ultrastructure, physiological function, and water permeability of oocytes during cryopreservation. We observed that the vitrified–warmed oocytes in the 10^{-9} M-MC group showed a lower incidence of early apoptosis than those in the NC group ($P<0.01$). In accordance with other studies, oocyte apoptosis is related to excessive ROS, increased intracellular Ca^{2+} concentrations, and mitochondrial dysfunction[42-45]. Our results indicate that MLT treatment can effectively inhibit the aggravation of early apoptosis in oocytes during cryopreservation, which is consistent with the report of Martín et al[46]. Ultrastructural damage is one of the most severe detrimental effects that are associated with cryopreservation[47]. Our results showed that oocytes in both the F group and 10^{-9} M-MC group maintained the normal ultrastructure (including mitochondria, CGs, microvilli, and vacuoles) and the permeability of the oolemma, thereby implying that MLT can effectively protect the ultrastructure of oocytes from cryoinjury and maintain the water permeability of the

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oolemma during cryopreservation. Mitochondria are the major sites of MLT synthesis and MLT functional targets[48]. MLT can maintain the normal structure, function, and distribution of mitochondria in oocytes during vitrification[49]. Hence, in our study, spherical or elliptical mitochondria showed the presence of more obvious cristae in their matrix in the MLT-treated group than in the NC group. Cryopreservation can cause a significant increase in the intracellular Ca^{2+} concentration, which promotes the exocytosis of premature CGs, leading to zona pellucida hardening, thereby reducing the developmental competence of oocytes after cryopreservation[50-52]. In experiment II, we observed that MLT treatment effectively prevented this abnormal exocytosis during cryopreservation. Moreover, a large number of vacuoles were observed in the vitrified–warmed oocytes of the NC group. Multiple vacuoles in the cytoplasm represent a nonspecific response to osmotic injury and/or cryoinjury[47]. Some researchers have confirmed that vacuolization is a morphological manifestation of the degradation process[53]. Our results showed that MLT treatment completely prevented vacuolization during cryopreservation. In the F group and the 10^{-9} M-MC group, numerous normal microvilli were distributed in the perivitelline space, whereas in the NC group, many severely degenerated microvilli were observed. It has been reported that the presence of degenerated microvilli in oocytes after cryopreservation indicates severe damage to the oolemma[54]. Therefore, our observations indicate that due to the effect of MLT, the oolemma structure of cryopreserved oocytes is well protected. During the vitrification–warming process, the oolemma is responsible for mediating the exchange of water and osmotic CPAs, which is defined as the permeability of the oolemma. However, permeability determines the cryotolerance of cells as it modulates various types of cryoinjury, especially through the formation of ice crystals[55-58]. Therefore, if the permeability of the oolemma cannot be effectively maintained during vitrification and warming, the formation of ice crystals inside and outside the oocyte will be inevitable and will directly damage the integrity of oolemma and intracellular organelles, even causing the oocyte to die after vitrification and warming. In the oolemma permeability experiment, our results showed that the oolemma of vitrified oocytes from the NC group had poorer hydraulic conductivity than those from the 10^{-9} M-MC and fresh groups (both $P<0.05$). Meanwhile, we also observed that the normalized volume of the NC group was more

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than 1 compared with the fresh group or the 10^{-9} M-MC group (Figure 5A). This may be the reason that the ultrastructure and oolemma of the oocytes in the NC group suffered severe damage during cryopreservation due to the lack of the protective effects of MLT, and the oolemma failed to effectively mediate water transport across the membrane in the further permeability experiment. For the oocytes from the 10^{-9} M-MC group almost no obvious damage was observed, which was comparable to fresh oocytes. Therefore, the normalized volume of the oocytes from the NC group was different from that of the10^{-9} M-MC group or the fresh group. In summary, the TEM and water permeability results of experiment II strongly suggest that MLT treatment effectively maintains the permeability of oolemma during cryopreservation.

Although the cryopreservation technology for human oocytes has been enhanced and has achieved a high survival rate, it still cannot be routinely applied in clinical practice because of the poor developmental competence of oocytes after cryopreservation[11,12]. A previous study has shown that MLT acts as a protective agent that can promote oocyte development[59]. In this study, we found that cryopreservation reduced the developmental competence of human oocytes, whereas MLT treatment significantly reversed this adverse outcome, especially for blastocyst formation rate, which increased from 11.11% in the NC group to 39.02% in the 10^{-9} M-MC group ($P<0.05$). Collectively, the results of experiment II indicate that MLT treatment can effectively improve the effect of cryopreservation in human oocytes.

To further analyze the mechanism of action of MLT (10^{-9} M) in improving the effect of cryopreservation in human oocytes, in experiment III, DEGs were screened and analyzed in viable oocytes after cryopreservation using sc-RNA sequencing technology. Interestingly, the expression of *AQP1*/*2*/*11* genes, which are a part of the AQP family and are related to cryotolerance[60], was upregulated in the 10^{-9} M-MC group compared with the NC group, whereas based on the subsequent confocal fluorescence staining results, the expression level of AQP1 protein in the 10^{-9} M-MC group was significantly increased compared to that in the NC group, and was comparable to that in the F group. These results indicate that MLT treatment can prevent the inhibitory effect of the vitrification procedure on the expression of AQP1 during cryopreservation. It has been

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reported that maintaining balanced water-solute exchange in cells has mainly been accomplished by AQPs during biological evolution[61]. AQPs, a small family of transmembrane channel proteins with molecular weights between 25 and 34 kD, allow water and solute transport across membranes[62]. A previous study found that AQP1 was located in the plasma membrane of mouse oocytes[55]. AQP1 is classified as an orthodox subtype of AQPs, which is permeable only to water. Even at lower temperatures, AQP1 can facilitate water transport across the oolemma in a channel-mediated manner[15,63]. Therefore, we speculate that MLT regulates the water permeability of oolemma through AQP1, thereby effectively preventing ice crystal formation during cryopreservation. In combination with the conclusion of experiment II, it can be concluded that MLT treatment can prevent the inhibitory effect of the vitrification procedure on AQP1 expression, thereby preventing ultrastructural damage caused by ice crystals. Some reports have examined these intracellular processes of MLT in oocytes[64-67]; however, the details remain unclear. The mechanism through which MLT regulates the nuclear expression of *AQP1* gene is unknown. Epigenetic modifications, such as DNA methylation and histone acetylation, are involved in regulating the expression of specific genes (such as *AQP1*) in oocytes. Hence, further research is needed to gain clarity on this mechanism.

In conclusion, MLT treatment can notably improve the effect of cryopreservation in human oocytes. The underlying mechanism of action of MLT is illustrated in Figure 9. The developmental competence of cryopreserved oocytes is most likely improved by suppressing oxidative stress, and MLT directly reduces ROS levels in oocytes to protect mitochondrial function in a non-receptor-mediated manner rather than in a receptor-mediated (Figure S1), thereby inhibiting the aggravation of early apoptosis. Furthermore, the developmental competence of cryopreserved oocytes is ameliorated through the maintenance of oolemma permeability to water. Hence, MLT may maintain the permeability of the oolemma by blocking the inhibitory effect of the vitrification procedure on AQP1 expression, thereby preventing ultrastructural damage in human oocytes.

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Competing interests

The authors declare that they have no competing interests.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1. A flowchart of the experimental design.

Figure 2. Effect of melatonin (MLT) treatment on the morphology of cryopreserved human oocytes from the no-MLT-treated cryopreservation (NC) group (0 M) and the MLT-treated cryopreservation (MC) group (10^{-11}, 10^{-9}, 10^{-7}, and 10^{-5} M). Fresh oocytes (F group) served as the control. Morphological changes were observed under an optical microscope: (1) granulation clusters and vacuoles in the cytoplasm of human oocytes (as indicated by the red arrows) in the NC group and 10^{-5} M-MLT subgroup; (2) a large perivitelline space in the 10^{-5} M-MLT subgroup; (3) a slightly rough cytoplasm in the 10^{-7} M-MLT subgroup and the 10^{-11} M-MLT subgroup; (4) uniform cytoplasm and normal morphology in the F group and the 10^{-9} M-MLT subgroup. Bar = 10 µm.

Figure 3. Effect of MLT treatment on mitochondrial function in cryopreserved human oocytes from the NC group (0 M) and the MC group (10^{-11}, 10^{-9}, 10^{-7}, and 10^{-5} M). Fresh oocytes (F group) served as the control. (A) Representative images of intracellular ROS levels after fluorescence staining with DCHFDA. Bar = 50 µm. (B) DCHFDA fluorescence intensity was quantified. Data were expressed as the mean ± SEM. *$P<0.05$ and ***$P<0.001$. (C) Representative fluorescence images of intracellular Ca^{2+} levels after staining with Fluo-4 AM. Bar = 50 µm. (D) Representative images of mitochondrial membrane potential after fluorescence staining with JC-1. Bar = 50 µm. (E) Fluo-4 AM fluorescence intensity was quantified. Data are expressed as the mean ± SEM. *$P<0.05$ and **$P<0.01$. (F) JC-1 fluorescence intensity was quantified. Data are expressed as the mean ± S.E.M. *$P<0.05$ and **$P<0.01$.

Figure 4. Effect of MLT treatment on the ultrastructural changes of cryopreserved human oocytes from the NC group (0 M) and the 10^{-9} M-MC group. Fresh oocytes (F group) served as the control. (A, B, C, D) Representative images of oocytes obtained via electron microscopy. (B, C) More vacuoles (Va) and degenerated mitochondria (M) were observed in the NC group than in the F group and 10^{-9} M-MC group. (D) Numerous long microvilli (Mi) were present on the oolemma of oocytes in the F group and 10^{-9} M-MC group. (A, D) Few cortical granules (CG) were

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discovered in perivitelline space of oocytes in the NC group. Bar = 5μm, 2μm, 1μm, and 1μm, respectively (A, B, C, D). Va: vacuoles; M: mitochondria; Mi: microvilli; CG: cortical granules.

Figure 5. Effect of MLT treatment on the permeability of oolemma in cryopreserved human oocytes from the NC group (0 M) and the 10^{-9} M-MC group. Fresh oocytes (F group) served as the control. (A) Change of oocyte volume with time (shrinkage and reswelling processes) in 10% ethylene glycol (EG)/M199 medium. (B) Hydraulic conductivity (L_P) was quantitated. Data are expressed as the mean ± SEM. The asterisks indicate statistically significant differences (*P<0.05). (C) EG permeability (P_{EG}) was quantitated. Data are expressed as the mean ± SEM. (D) Change of oocyte volume with time (shrinkage process) in 10% EG/M199 medium. (E) Oocytes' minimum value of the normalized cell volume (V/V_0 min) was determined. Data are expressed as the mean ± SEM. The asterisks indicate statistically significant differences (*P<0.05, ***P<0.001).

Figure 6. Effect of MLT treatment on early apoptosis and developmental competence in cryopreserved human oocytes from the NC group (0 M) and the 10^{-9} M-MC group. Fresh oocytes (F group) served as the control. (A) Representative images of early apoptosis in oocytes after fluorescence staining with Annexin-V. The green fluorescence signal observed in the membrane and zona pellucida represents early apoptosis in oocytes (as shown in the NC group), whereas this signal is observed only in the zona pellucida of normal oocytes (as shown in the F group and the 10^{-9} M-MC group). Bar = 50 μm. (B) Data are expressed as the mean ± SEM. The asterisks indicate statistically significant differences (**P<0.01, ***P<0.001). (C) Representative images consisting of two pronucleus (2PN), 4-cell, 8-cell, and blastocysts captured with a Time-Lapse microscope.

Figure 7. Effect of MLT treatment on the global gene expression characteristics of cryopreserved human oocytes with the NC group (0 M) and the 10^{-9} M-MC group. Fresh oocytes (F group) served as the control. (A) The histogram shows the differentially expressed genes (DEGs) in the comparison groups. (B) The heat map shows the differences in the mRNA expression levels of aquaporin (*AQP*) genes in the comparison groups. (C) The heat map shows the expression levels

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of *AQP1*, *AQP2*, and *AQP11* genes in the comparison groups. Gene Ontology (GO, http://www.geneontology.org/) enrichment analysis of the *AQP1*, *AQP2*, and *AQP11* genes in (D) the molecular function category, (E) the biological process category, (F) the cellular component category.

Figure 8. Effect of MLT treatment on the protein expression levels of AQP1, AQP2 and AQP11 in cryopreserved human oocytes from the NC group (0 M) and the 10^{-9} M-MC group. Fresh oocytes (F group) served as the control. (A, B, C) Representative images of oocytes obtained after immunofluorescence staining. Bar = 50 μm. (D, E, F) Fluorescence intensity was quantified. Data are expressed as the mean ± SEM. The asterisks indicate statistically significant differences (**$P<0.01$).

Figure 9. The effect and mechanism of 10^{-9} M MLT treatment in cryopreserved human oocytes. MLT entered the cytoplasm through the cell membrane in a non-receptor-mediated manner (**Figure S1**). It was found that: (1) 10^{-9} M MLT treatment improved mitochondrial function by suppressing oxidative stress, decreasing intracellular ROS and Ca^{2+} levels, and maintaining normal MMP, thus preventing oocyte apoptosis. (2) There were two ways (lipid bilayer and aquaporins-mediated process) of water transmembrane transport. 10^{-9} M MLT treatment may maintain the permeability of the oolemma by preventing the inhibitory effect of the vitrification procedure on AQP1 expression.

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Table 1. Effect of MLT treatment on the developmental competence of cryopreserved human oocytes.

	F-group	**NC-group**	**10^{-9}M-MC-group**
No. of matured oocytes	50	55	51
Survival rate (%)	--	87.27% (48/55)	94.12% (48/51)
Fertilization rate (%)	90.00% (45/50)[a]	62.50% (30/48)	89.58% (43/48)[b]
Cleavage rate (%)	95.56% (43/45)	90.00% (27/30)	95.35% (41/43)
High-quality cleavage embryo rate (%)	67.44 (29/43)[a]	25.93% (7/27)	65.85% (27/41)[b]
Blastocyst rate (%)	53.49% (23/43)[c]	11.11% (3/27)	39.02% (16/41)[d]

Data were analyzed using the Chi-square or Fisher Exact test. [a]$P<0.01$, [b]$P<0.01$, [c]$P<0.001$ and [d]$P<0.05$ compared with the NC group, respectively.

A cleavage embryo with 6–8 equal-sized blastomeres, as well as no fragments or <20% fragmentation, was designated as a high-quality cleavage embryo. Survival rate is based on the number of survived oocytes / the number of matured oocytes.

Fertilization rate is based on the number of fertilized oocytes / the number of survived oocytes.

Cleavage rate is based on the number of cleavage embryos / the number of fertilized oocytes.

High-quality cleavage embryo rate is based on the number of high-quality cleavage embryos / the number of cleavage embryos.

Blastocyst rate is based on the number of blastocysts / the number of cleavage embryos.

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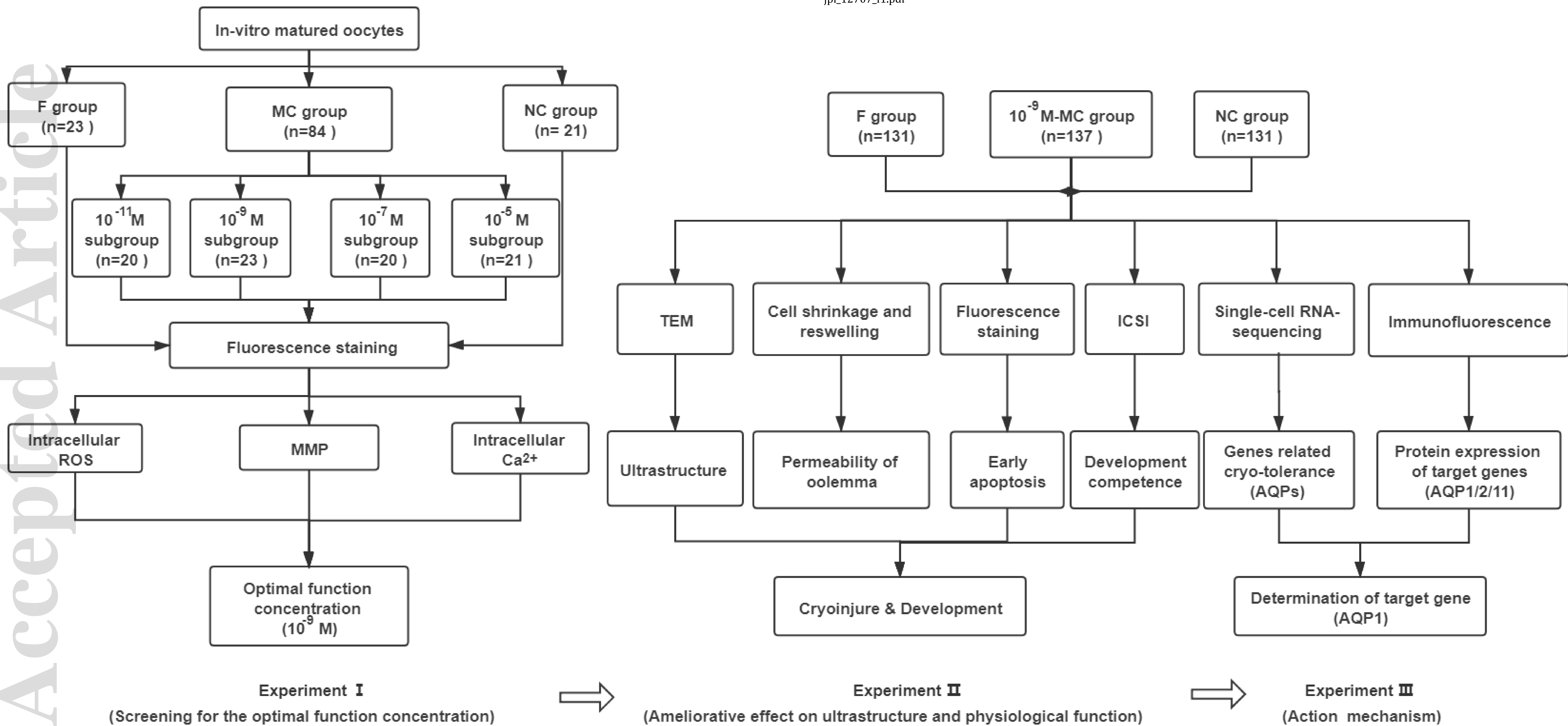

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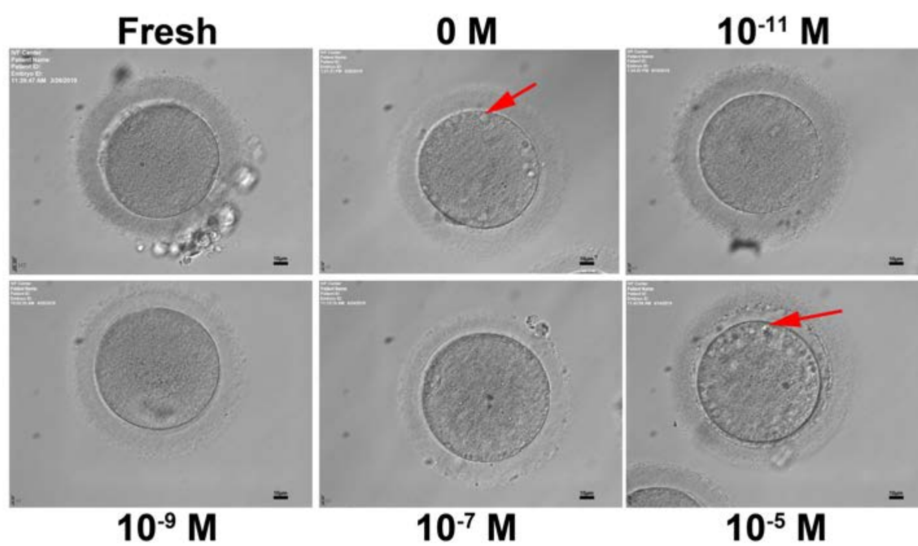

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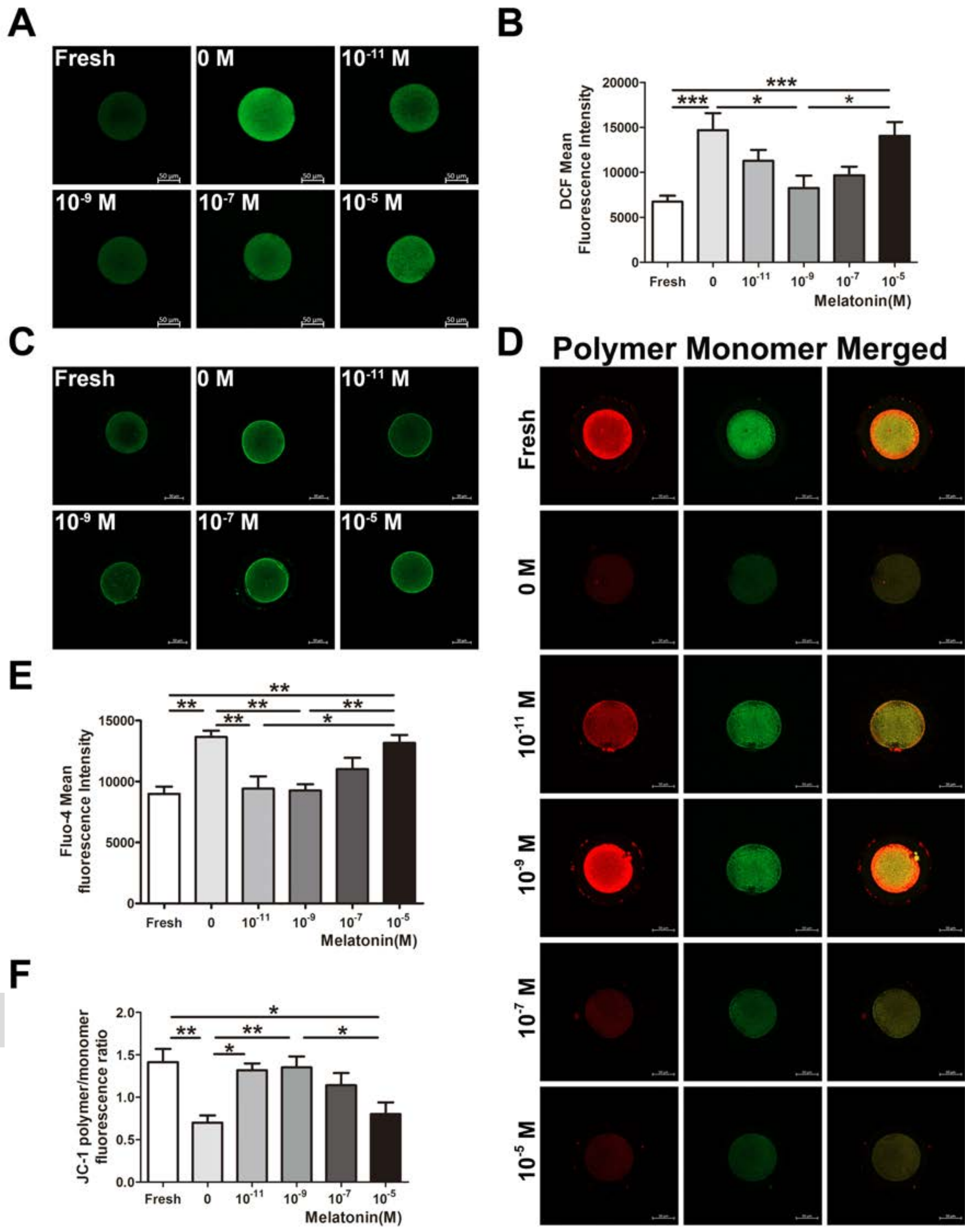

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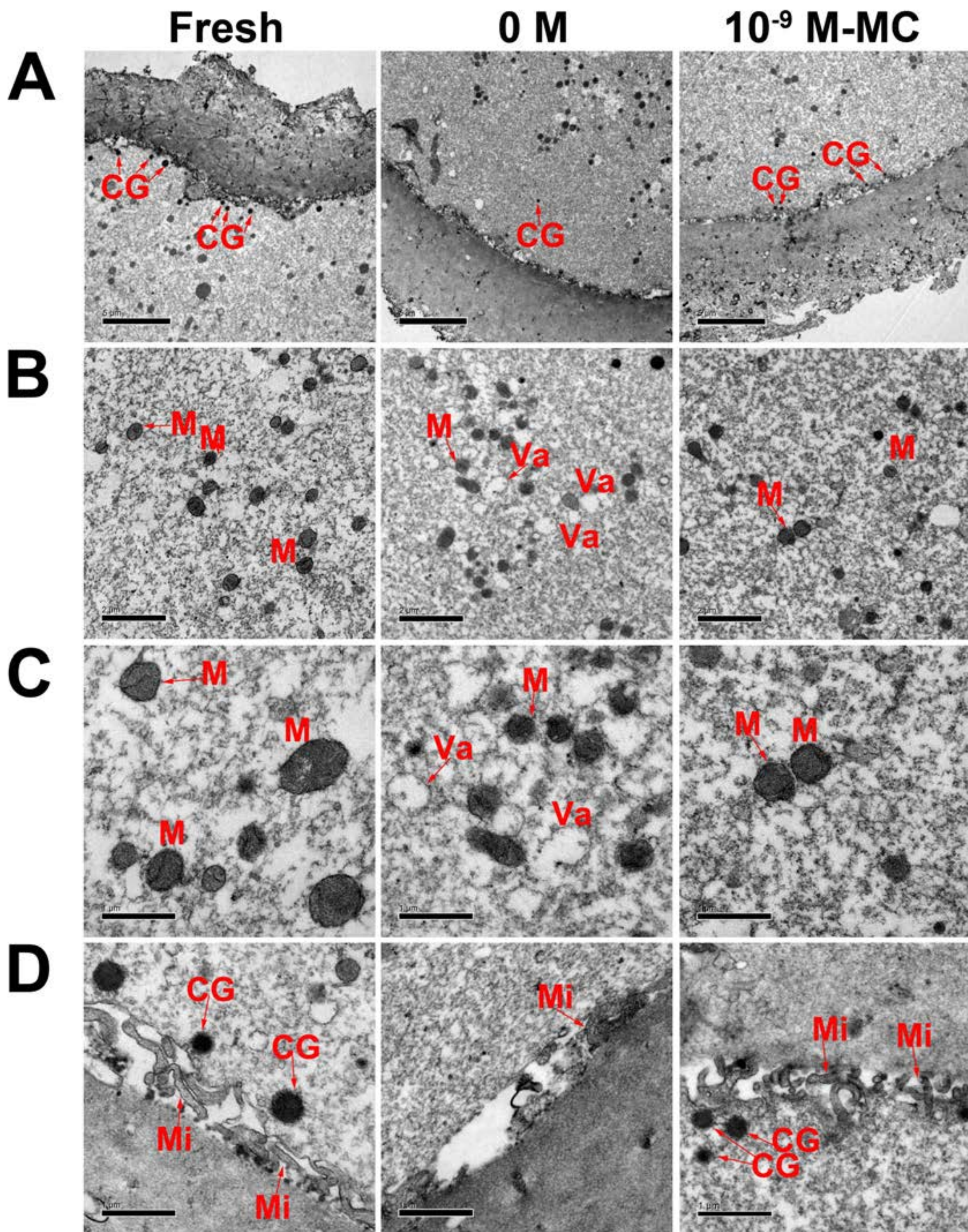

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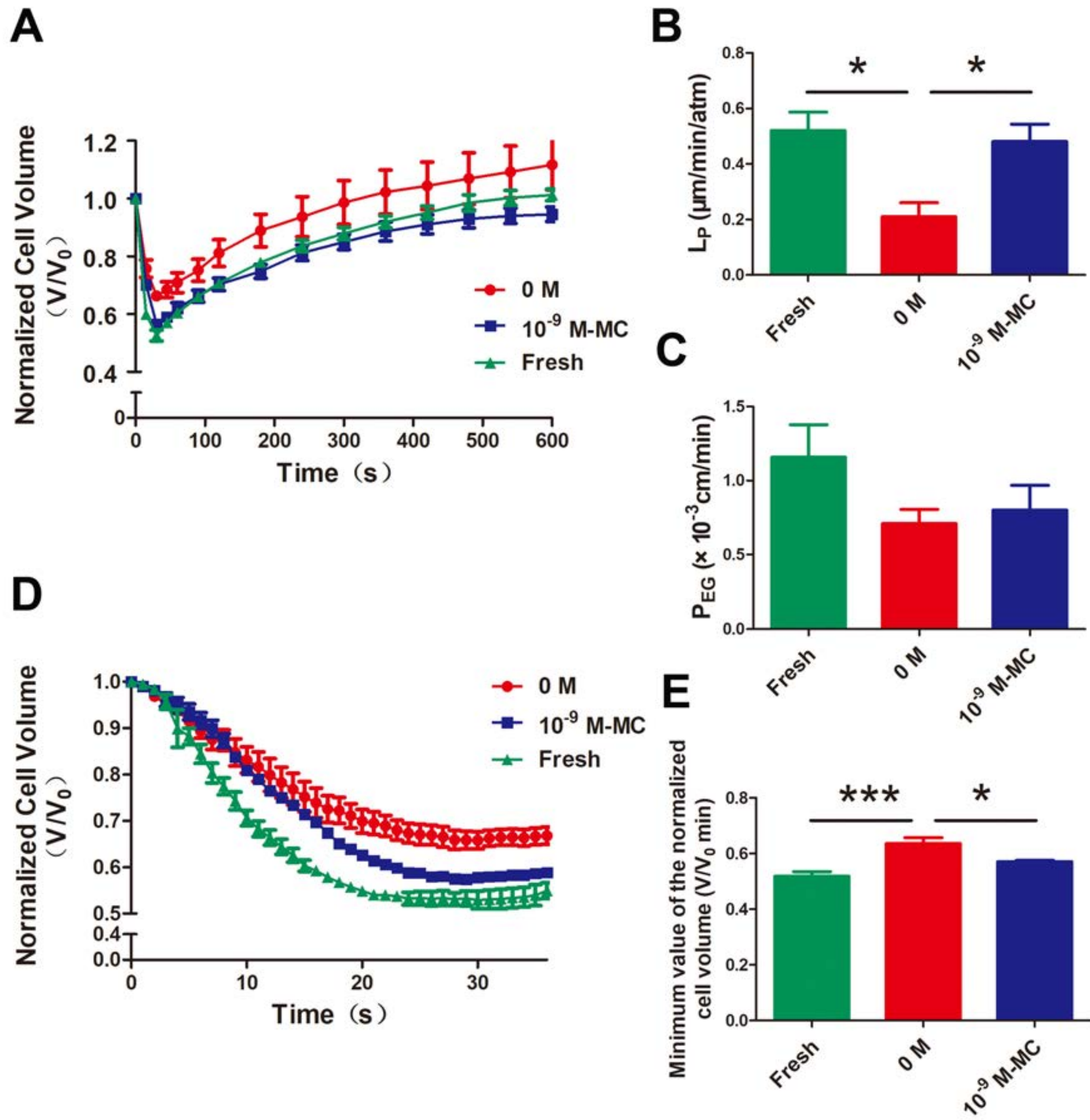

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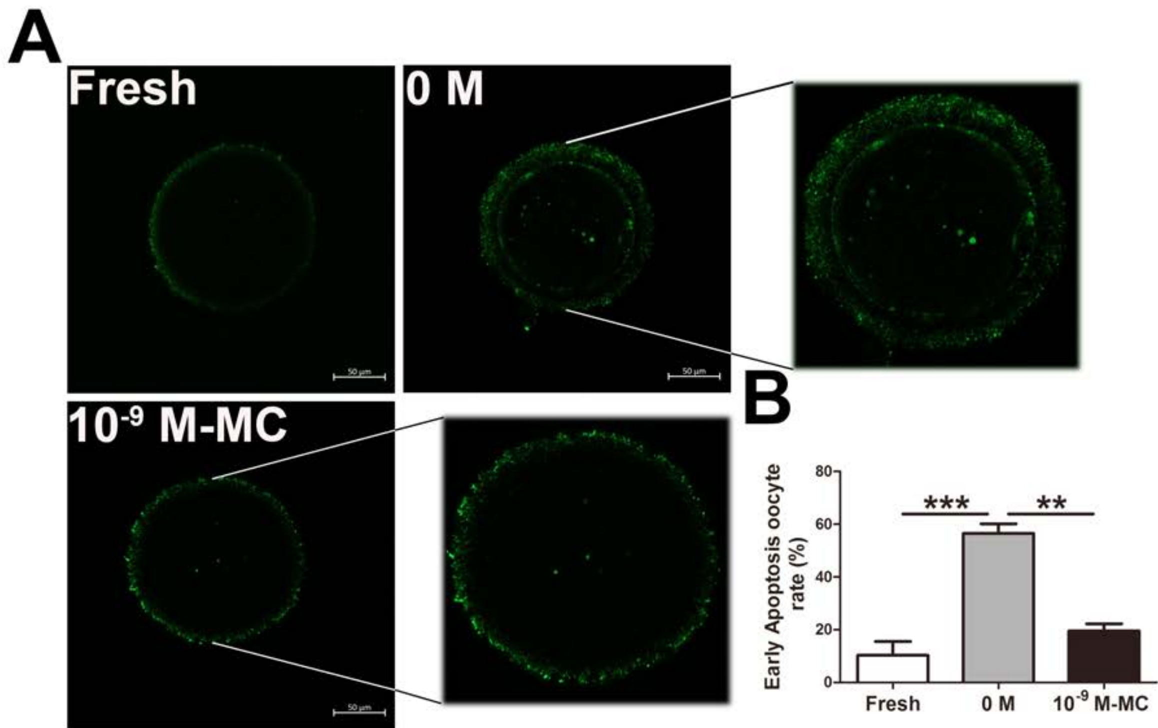

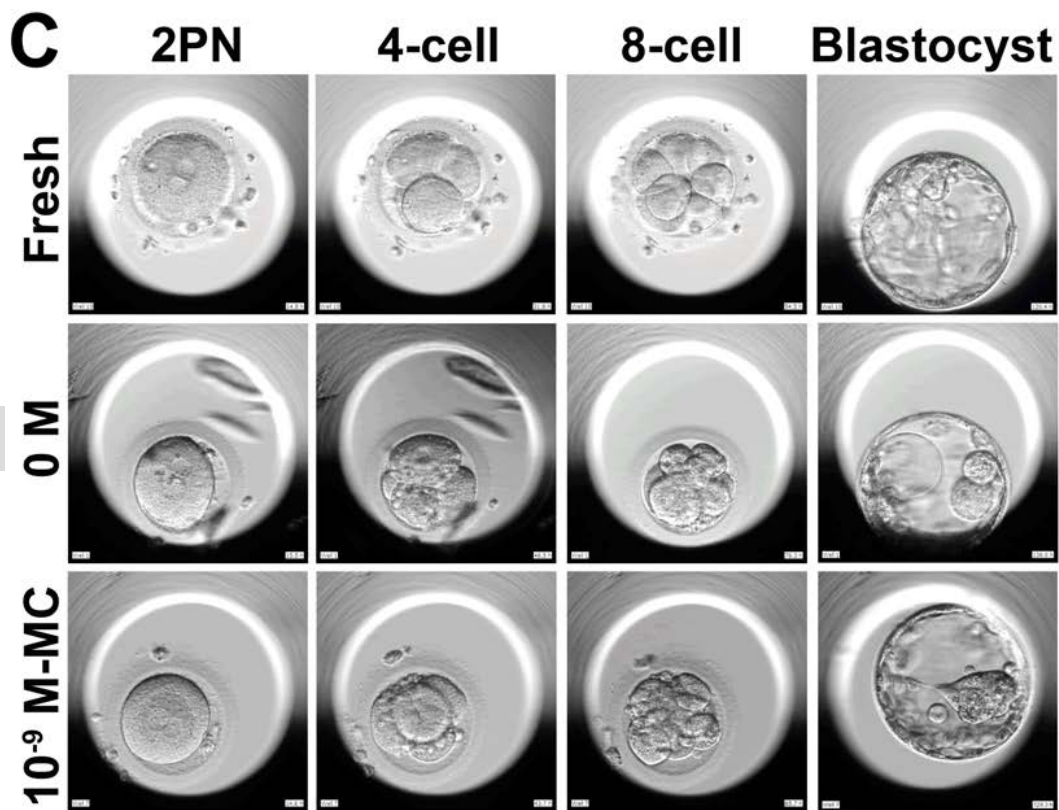

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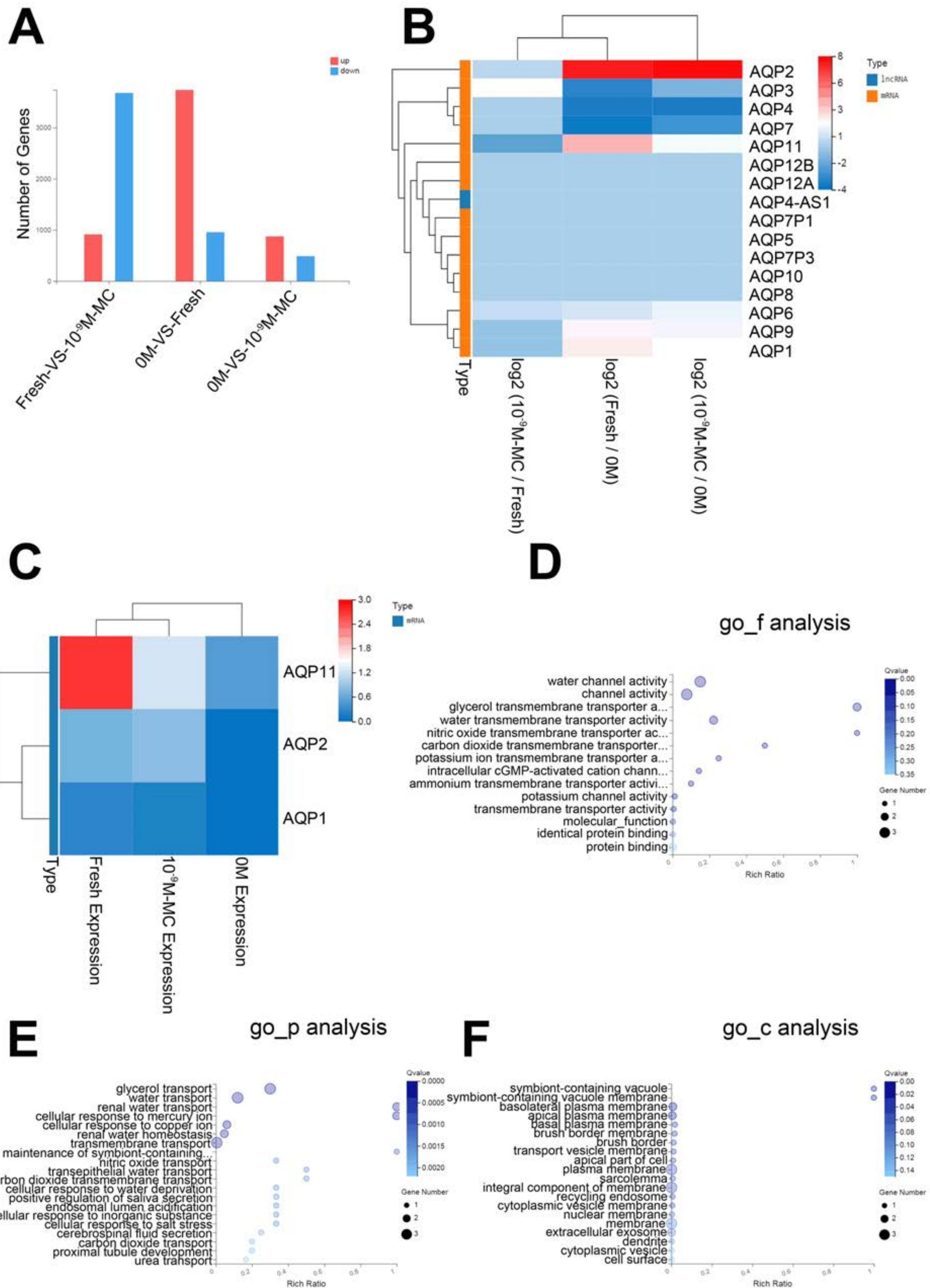

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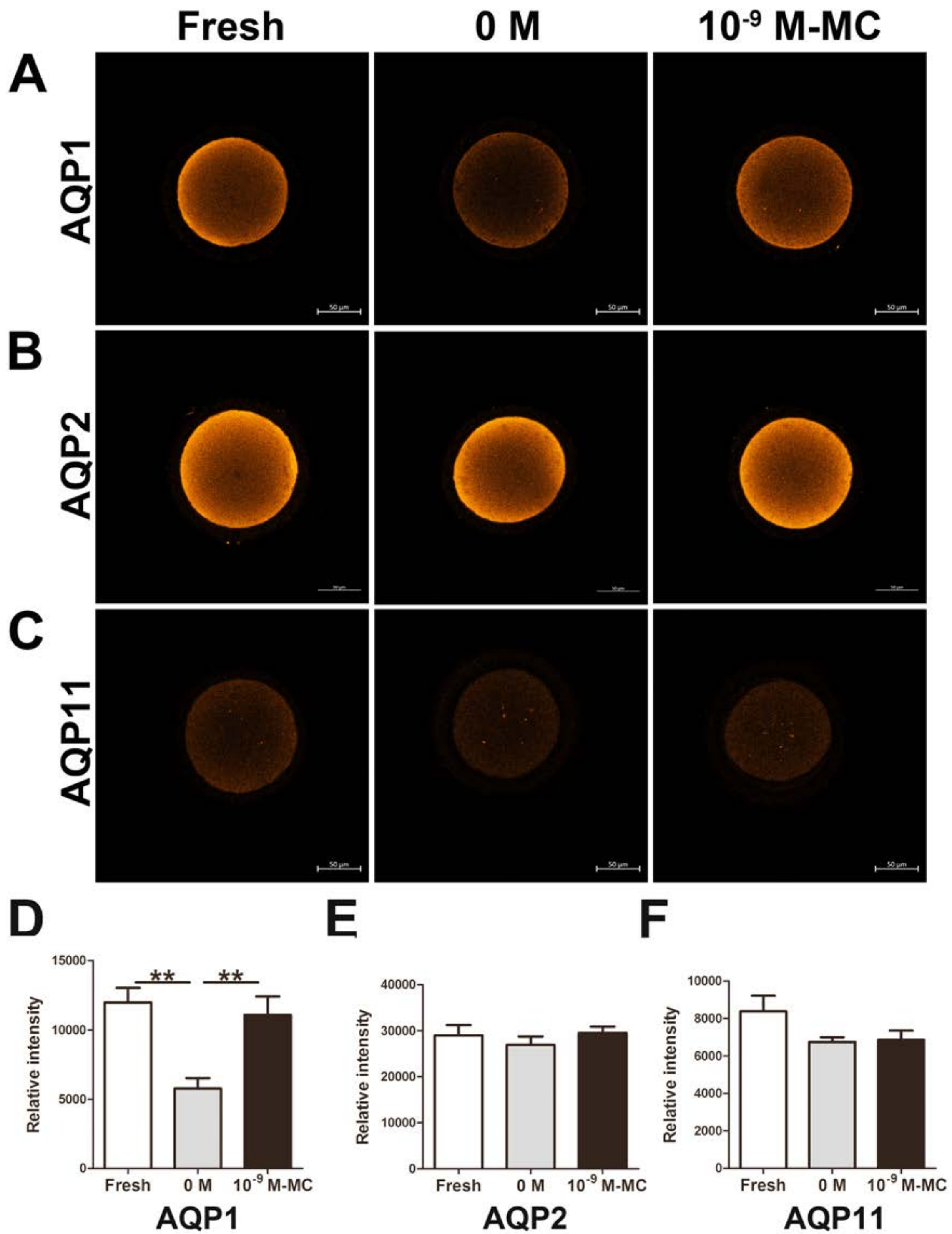

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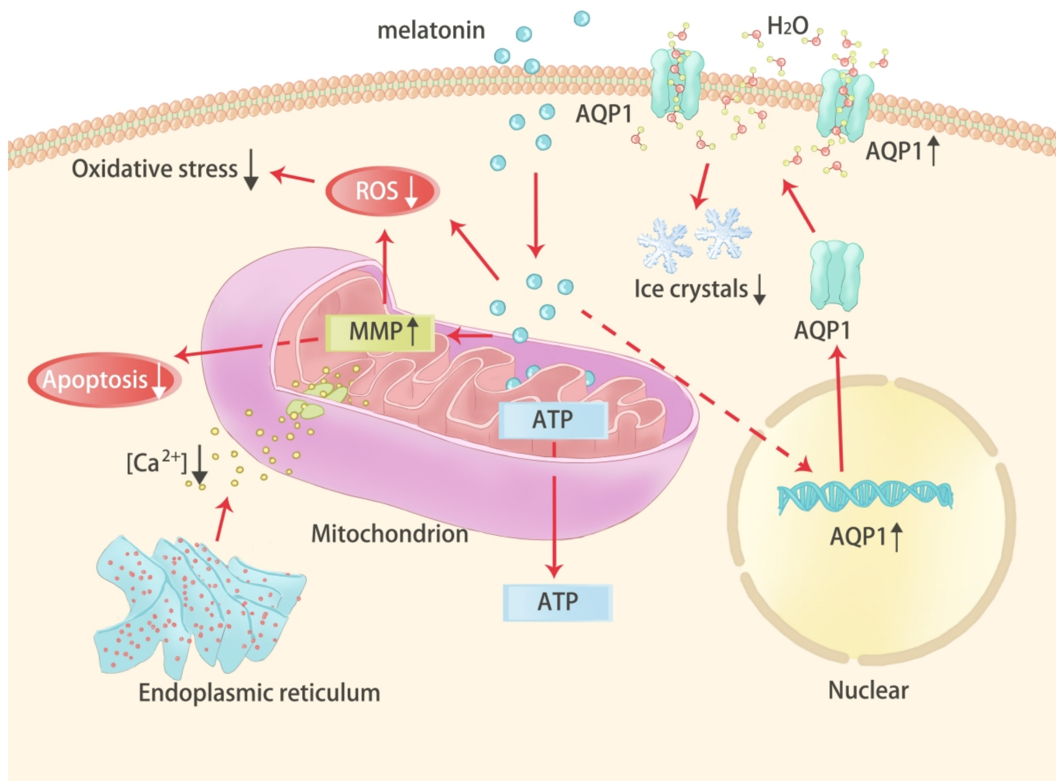

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