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Human placenta-derived mesenchymal stem cells inhibit apoptosis of granulosa cells induced by IRE1 $\alpha$  pathway in autoimmune POF mice

**Running Head:** hPMSCs inhibit GCs apoptosis in POF mice

Hongxing Li <sup>1\*</sup> · Wei Zhao <sup>1\*</sup> · Li Wang <sup>2</sup> · Qianqian Luo <sup>3</sup> · Na Yin <sup>1</sup> · Xueyan Lu <sup>1</sup> · Yun Hou <sup>1</sup> · Jingjing Cui <sup>2</sup> · Hongqin Zhang <sup>1</sup>

<sup>1</sup>Department of Histology and Embryology, Binzhou Medical University, Yantai 264003, Shandong, China

<sup>2</sup>Department of Obstetrics and Gynecology, Binzhou Medical University Hospital, Yantai 264100, Shandong, China

<sup>3</sup>Morphology Laboratory, Binzhou Medical University, Yantai 264003, Shandong, China

\* Hongxing Li and Wei Zhao contributed equally to this work

**Corresponding author:** Hongqin Zhang, Department of Histology and Embryology, Binzhou Medical University, No. 346 Guanhai Road, Laishan

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District, Yantai 264003, Shandong, PR China; Jingjing Cui, Department of Obstetrics and Gynecology, Binzhou Medical University Hospital, No. 717 Jinyu Street, Muping District, Yantai 264100, Shandong, PR China.

**Email:** hqzbmu@163.com; byfycjj@126.com

**Tel:** +86-13583511020; +86-15054395517

### **Abbreviations**

AMH: Anti-Müllerian hormone; ANOVA: One-way analysis of variance; ATF6: Activating transcription factor 6; AZPAb: Anti-zona pellucida antibody; Bcl-2: B cell lymphoma-2; BIM: Bisindolylmaleimide; CHOP: C/EBP-homologous protein; DAB: Diaminobenzidine; DAPI: 4', 6-diamidino-2-phenylindole; DMEM: Dulbecco's modified Eagle's Medium; E<sub>2</sub>: Estradiol; ECL: Enhanced chemiluminescence; ELISA: Enzymelinked immunosorbent assay; ER: Endoplasmic reticulum; FCA: Freund's complete adjuvant; FCM: Flow cytometry; FIA: Freund's incomplete adjuvant; FITC: Annexin-V-fluorescein isothiocyanate; FSH: Follicle stimulating hormone; GC: Granulosa cell; GRP78: 78kDa glucose-regulated protein; HPLC: High-performance liquid chromatography; hPMSCs: Human placenta mesenchymal stem cells; IRE1 $\alpha$ : Inositol-requiring enzyme 1 $\alpha$ ; JNK: c-Jun N-terminal kinase; LH: Luteinizing hormone; MSCs: Mesenchymal stem cells; OD: Optical density; PBS:

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Phosphate-buffered saline; PERK: PKR-like endoplasmic reticulum kinase;  
PMSF: Phenylmethanesulfonyl fluoride; POF: Premature ovarian failure; PVDF:  
Polyvinylidene difluoride; pZP3: Zona pellucida 3 peptide; SDS-PAGE: Sodium  
dodecyl sulfate polyacrylamide gel electrophoresis; TBST: Tris-buffered saline  
containing 0.1% Tween 20; TUNEL: TdT-mediated dUTP Nick-End Labeling;  
UPR: Unfolded protein response; XBP1: X-box binding protein 1

### **Abstract**

Previous studies have shown that the ovarian failure in autoimmune-induced premature ovarian failure (POF) mice could be improved by the transplantation of human placenta-derived mesenchymal stem cells (hPMSCs); however, the protective mechanism of hPMSCs transplantation on ovarian dysfunction remains unclear. Ovarian dysfunction is closely related to the apoptosis of granulosa cells (GCs). To determine the effects of hPMSCs transplantation on GCs apoptosis, an autoimmune POF mice model was established with zonapellucida glycoprotein 3 (ZP3) peptide. It is reported that the inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ) and its downstream molecules play a central role in the endoplasmic reticulum (ER) stress-induced apoptosis pathway. So the aim of this study is to investigate whether hPMSCs transplantation attenuated GCs apoptosis via inhibiting ER stress IRE1 $\alpha$  signaling pathway. The ovarian dysfunction, follicular dysplasia and GCs apoptosis were observed in the POF mice. And the IRE1 $\alpha$  pathway was

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activated in ovaries of POF mice, as demonstrated by, increased X-box binding protein 1 (XBP1), up-regulated 78kDa glucose-regulated protein (GRP78) and Caspase-12. Following transplantation of hPMSCs, the ovarian structure and function were significantly improved in POF mice. In addition, the GCs apoptosis were obviously attenuated and IRE1 $\alpha$  pathway was significantly inhibited. Transplantation of hPMSCs suppressed GCs apoptosis induced by ER stress IRE1 $\alpha$  signaling pathway in POF mice, which might contribute to the hPMSCs transplantation-mediating ovarian function recovery.

**Keywords:** Premature ovarian failure, Human placenta-derived mesenchymal stem cell, IRE1 $\alpha$ , Endoplasmic reticulum stress, Granulosa cells, Apoptosis

## 1. Introduction

Premature ovarian failure (POF) is a syndrome clinically defined by failure of the ovary before the age of 40 (Hoek et al., 1997). It is a disease characterized by anovulation, genital atrophy, estrogen deficiency and elevated gonadotropin levels. About 1% of women below the age of 40 and 0.1% under the age of 30 will develop POF (Fenton, 2015). Depending upon the age at diagnosis, many factors may result in the pathogenesis of POF such as genetic and autoimmune factors. About 5-30 % of POF cases result from an autoimmune etiology (Silva et al., 2014; Ostensen et al., 2011). At present, the clinical treatment strategies of

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POF cannot effectively improve the function of damaged ovaries (Hewlett and Mahalingaiah, 2015). Given the limitation of conventional treatment, scientists are actively looking for other therapeutic measures, such as stem cell therapy.

Recently, transplantation of mesenchymal stem cells (MSCs) has made great progress in the treatment of POF in animal studies (Li et al., 2018; Badawy et al., 2017). Among MSCs, the human placenta-derived mesenchymal stem cells (hPMSCs) are widely investigated for the treatment because the hPMSCs have low immunogenicity, small chance of virus infection and no adverse effects on the donor (Parolini et al., 2008). Yin et al. has shown that the transplantation of hPMSCs into pZP3-induced autoimmune POF mice could promote the recovery of abnormal ovarian function (Yin et al., 2018), which showed a therapeutic effect on POF mice. However, the mechanisms of hPMSCs on the improvement of ovarian function are still not well understood. This makes it a challenge to treat the POF at early stage.

Studies have shown that granulosa cells (GCs) play an important role in supporting ovarian function (Gao et al., 2014; Kuo et al., 2011). The proliferation and differentiation of GCs are the basic conditions for continuous development of ovarian follicles. In addition, GCs are the important source of estrogen and progesterone. Therefore, GCs apoptosis may cause a decrease in the number of

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follicles, reduced level of estrogen, and eventually lead to ovarian failure (Fu et al., 2017).

Endoplasmic reticulum (ER) stress is reported a signaling pathway to induce cell apoptosis (Hetz et al., 2015). ER is an important organelle for cellular protein processing and synthesis, maintaining the stability of cellular environment. Various changes in cell, such as hypoxia, stress or long-term stimulation of physical and chemical conditions can cause the accumulation of unfolded or misfolded proteins in the lumen of ER, which triggers unfolded protein response (UPR) (Moore and Hollien, 2012). In response to ER stress, UPR is able to induce the activation of three signaling pathways including activating transcription factor 6 (ATF6) pathway, protein kinase RNA (PKR)-like ER kinase (PERK) pathway, and inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ) pathway.

IRE1 $\alpha$  is an ER transmembrane sensor capable of regulating cell fate. Upon activation, IRE1 $\alpha$  induces signaling transduction events that alleviate the accumulation of unfolded/misfolded proteins in the ER by increasing expression of ER molecular chaperone 78 kDa glucose-regulated protein (GRP78) (Sano and Reed, 2013). As reported, pathogenesis of many autoimmune diseases is related to cell apoptosis caused by unfolded/misfolded proteins accumulation, including multiple sclerosis, rheumatoid arthritis and inflammatory bowel disease (Hetz et al., 2013). Studies have confirmed that maintaining ER Ca<sup>2+</sup> homeostasis of GCs

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is important for ovarian follicle development (Sun et al., 2018). In addition, previous studies have shown that transplantation of human placenta-derived mesenchymal stem cells (hPMSCs) improved the ovarian function of POF mice and reduced the expression of Caspase-3 activation (Yin et al., 2018). So based on the above, in the present study, we aimed to explore the effect of hPMSCs transplantation on the GCs apoptosis in an autoimmune POF mice model, through regulation of the expression of IRE1 $\alpha$  pathway of ER stress in ovaries.

## **2. Materials and methods**

### **2.1 Experimental animals**

Female BALB/c mice of 6-8 weeks old, weighing 18-22 g, were obtained from Jinan Pengyue Experimental Animal Breeding Co, Ltd (China). All animals were housed in SPF Laboratory of Experimental Animal Center of Binzhou Medical University, with ad libitum access to water and standard pellet feed. All procedures were approved by the Institutional Animal Care and Use Committee of Binzhou Medical University, and the study was carried out in accordance with the National Research Council Guide for Care and Use of Laboratory Animals.

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## **2.2 Isolation and culture of human placenta-derived mesenchymal stem cells (hPMSCs)**

Human placenta tissues were obtained from healthy mothers who underwent the caesarean section. And during pregnancy, the women have been tested negative for HIV-I, hepatitis B and hepatitis C following informed consent. The hPMSCs were isolated and cultured as previously described (Yin et al., 2018; Yin et al., 2018). The cell membrane and intracytoplasmic molecular markers of hPMSCs were examined using flow cytometry to confirm the phenotype of hPMSCs as we previously described (Yin et al., 2018; Yin et al., 2018).

## **2.3 Premature ovarian failure (POF) mice model**

The zonapellucida glycoprotein 3 (ZP3) peptide was used to establish the autoimmune POF mice model. The ZP3 peptide (pZP3) was synthesized by an automatic peptide synthesizer (Hangzhou Economic & Technological Development Zone, China). First, pZP3 was dissolved in sterile, distilled water at 1 mg/ml. The pZP3 solution was then mixed with Freund's complete adjuvant (FCA; Mycobacterium tuberculosis H37RA strain, 0.16 mg/mouse; Sigma, USA) or Freund's incomplete adjuvant (FIA; Mycobacterium tuberculosis H37RA strain, 0.16 mg/mouse; Sigma) with a ratio of 1:1 before use.



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Mice ( $n = 79$ ) were randomly divided into control group ( $n = 14$ ) and pZP3 group ( $n = 65$ ). The control animals were normal mice. The mice given pZP3 were first subcutaneously injected with 0.15 ml immune reagent FCA at multiple sites in abdomen and hind footpads, and then injected with 0.15 ml immune-strengthening reagent FIA at the same sites 14 days later. After injecting the immune-strengthening reagent for 7 days, the tail vein blood of all mice was extracted for qualitative detection of anti-pZP3 antibody (AZPAb). The autoimmune response was confirmed by the presence of AZPAb. Then the mice with positive expression of AZPAb were screened for subsequent experiments. The selected AZPAb (+) mice in pZP3 group were randomly divided into three groups: POF group ( $n = 20$ ), POF + hPMSCs transplantation group ( $n = 20$ ) and POF + vehicle group ( $n = 20$ ). The mice in the POF group did not receive any other treatment after the modeling procedure.  $1 \times 10^6$  hPMSCs at sixth passages were slowly injected into the mice of POF + hPMSCs transplantation group via the tail vein one week after modeling (Yin et al., 2018; Park et al., 2012). For comparison, the same volume of phosphate buffered-saline (PBS) was injected into POF mice as vehicle control at the same time point.

#### **2.4 Determination of the establishment of autoimmune POF mice model**

After the mice were administered with the immune-strengthening reagent for one week, the blood was collected by tail vein puncture. The levels of estradiol ( $E_2$ ),

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follicle stimulating hormone (FSH) and luteinizing hormone (LH) in serum were measured by Enzyme-Linked Immunosorbent Assay (ELISA) kits (Lengton) according to manufacturer's instruction. The data were used to confirm the establishment of autoimmune POF mice model.

### **2.5 Ovarian morphology examination**

Ovaries of mice were collected 2 weeks after the hPMSCs transplantation, fixed in 4 % paraformaldehyde in phosphate buffer for 24 h, and then embedded in paraffin. Serial sections (4  $\mu$ m) were prepared and stained with H&E for morphological studies. The morphological structure of ovary was examined under biological light microscope (Olympus BX53, Japan) by two independent observers who were blind to the experimental data, and the images of ovary were taken. According to the accepted criteria described previously (Pedersen and Peters, 1968), the follicle at different stages was classified as primordial, primary, secondary, and atretic follicles. The primary follicles and secondary follicles are developing follicles (Myers et al., 2004).

### **2.5 TUNEL analysis**

For terminal transferase dUTP nick-end labelling (TUNEL), the apoptotic GCs in ovaries were detected using the In Situ Cell Death Detection Kit, Fluorescein (Catalog No. 11684795910; Roche Applied Science, Switzerland). Briefly, the

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ovarian sections were incubated in proteinase K working solution for 30 min at 37 °C. Then the slides were incubated with TUNEL reaction mixture for 1h at 37 °C followed by rinsing with 0.01 M PBS for three times. The nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI; Beyotime, China) for 5 min at room temperature. The sections were imaged using a fluorescence microscope (Leica, Germany), and the number of TUNEL-positive GCs was counted. The percentage of TUNEL-positive GCs in total cells was calculated as apoptosis index.

### **2.7 Immunohistochemical staining**

Immunohistochemical staining was performed on the ovarian sections. Ovarian sections were obtained as above. Briefly, the sections were incubated with the primary antibody: polyclonal rabbit anti-IRE1 $\alpha$  (1:200; Abcam, British), polyclonal rabbit anti-XBP1 (1:200; Proteintech, China), polyclonal rabbit anti-GRP78 (1:100; Proteintech, China), and polyclonal rabbit anti-Caspase-12 (1:200; Proteintech, China). Then the sections were incubated with corresponding biotinylated secondary antibody anti-rabbit IgG (Gene Tech, China), and the immunoreactivity was visualized with diaminobenzidine (DAB; Gene Tech).

### **2.8 Western blotting**

The mice were sacrificed 2 weeks following the hPMSCs transplantation. And then the bilateral ovaries were separated, washed with ice-cold PBS, and

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homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer with 1 mM PMSF (Beyotime Biotechnology, China). Protein concentrations were determined by the BCA protein assay kit (Beyotime Biotechnology). Equal amounts of protein were resolved by SDS-PAGE electrophoresis and transferred onto PVDF membranes (BioRad) by electroblotting. Briefly, the membranes were separately incubated with the following rabbit polyclonal antibodies: IRE1 $\alpha$  (1:500; Abcam), XBP1 (1:500; Proteintech), GRP78 (1:500; Proteintech), Caspase-12 (1:200; Proteintech) and  $\beta$ -actin (1:1000; Abcam). Then the membranes were incubated with horseradish peroxidase- conjugated secondary antibody (anti-rabbit IgG HRP, 1:5000; Santa Cruz, USA). Immunoblots were detected using enhanced chemiluminescence (ECL; Thermo Scientific, USA) and band intensities were calculated using Image J.

## 2.9 Statistical analysis

The results were analyzed with SPSS version 17.0 (SPSS, Inc., an IBM Company, Chicago, USA). Data were presented as means  $\pm$  standard deviation (SD). The statistical differences among groups were compared by one-way analysis of variance (ANOVA), followed by Tukey post-hoc multiple comparison test (Tukey's test). A  $P < 0.05$  value was considered statistically significant different with differences.

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### 3. Results

#### 3.1 The effects of hPMSCs transplantation on hormone secretion in POF mice

The effects of hPMSCs transplantation on the secretion of E<sub>2</sub>, FSH and LH were investigated. Compared with the control group, the serum levels of E<sub>2</sub> in POF group and POF + vehicle group were significantly decreased (Figure 1A;  $P < 0.01$ ), and the levels of FSH and LH were significantly increased (Figure 1B, C;  $P < 0.01$ ). However, there was no apparent difference in serum levels of E<sub>2</sub>, FSH and LH between the POF group and POF + vehicle group. The reduced sensitivity of follicles to gonadotropins suggests the ovarian dysfunction and successful establishment of POF animal model.

As shown in Figure 1, following hPMSCs transplantation in POF mice, the serum level of E<sub>2</sub> were significantly elevated ( $P < 0.01$ ), the levels of FSH and LH were decreased ( $P < 0.01$ ), compared with the POF group and POF + vehicle group. Based on the results from the changes of endocrine hormone (E<sub>2</sub>, FSH and LH), it suggested that hPMSCs transplantation affected the hormone regulation and promoted the recovery of ovarian function in POF mice.

#### 3.2 Follicular development in POF mice following hPMSCs transplantation

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The morphology of ovaries was examined by H&E staining. In control group, many healthy developing follicles and regular arrangement of GCs were examined in ovaries (Figure 2a, e). In contrast, the developing follicles decreased and the atretic follicles increased, the arrangement of GCs became irregular in the POF group and POF + vehicle group (Figure 2b, d, f, h). After hPMSCs transplantation for 2 weeks, it was observed that the developing follicles were increased, and the atretic follicles were reduced in POF mice (Figure 2c, g). Taken together, these results indicated that hPMSCs transplantation might be effective to promote the development and survival of follicles in injured ovaries.

### **3.3 Transplantation of hPMSCs suppressed the apoptosis of GCs in POF mice**

TUNEL analysis was performed to assess the cellular apoptosis in ovarian tissues in each group. As shown in Figure 3, few TUNEL-positive GCs were detected in control group. In contrast, the number of apoptotic GCs was much higher in the POF group and POF + vehicle group ( $P < 0.01$ ). The percentage of TUNEL-positive GCs in each group was illustrated in Figure 3B. The results suggested that the excessive apoptosis of GCs might be the cause of follicular dysplasia in POF mice. Following hPMSCs transplantation, the number of TUNEL-positive GCs was significantly decreased in ovaries of POF mice ( $P < 0.01$ ).

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### 3.4 The hPMSCs transplantation inhibited the activation of IRE1 $\alpha$ apoptosis pathway in POF mice

To determine whether IRE1 $\alpha$  signaling pathway was involved in ovarian function recovery after hPMSCs transplantation, the immunohistochemical staining of IRE1 $\alpha$  pathway was conducted on ovarian tissues. As shown in Figure 4, the expression of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12 were all located in the cytoplasm of GCs. In control group, the immunostaining of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12 all showed less expressed. Compared with the control group, the expression of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12 in ovaries were much higher in the POF group and POF + vehicle group ( $P < 0.01$ ). After hPMSCs transplantation, the positive expression of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12 were significantly inhibited in ovaries of POF mice ( $P < 0.01$ ).

Next, the protein expression of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12 in ovaries was quantitated with Western blotting, as shown in Figure 5. The data indicated that the expression of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12 protein were very low in control group. Compared with the control group, the expression of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12 all showed significant increase ( $P < 0.01$ ) in the POF group and POF + vehicle group. Following hPMSCs transplantation, the protein expressions were all significantly decreased ( $P < 0.01$ ). These data suggest that the transplantation of hPMSCs may attenuate the

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activation of IRE1 $\alpha$  mediated ER stress apoptosis pathway and promote the recovery of ovarian function in POF mice.

#### **4. Discussion**

In the present study, the ZP3 peptide was used to establish the autoimmune POF mice model. The presence of anti-autoantibodies can induce the activation of autoimmune T cell response and lead to severe organ failure (Luo et al., 1993). In addition, anti-ZP antibody (AZPAb) is involved in follicular development and may be one of the causes of autoimmune POF (Takamizawa et al., 2007).

Therefore, we used the expression of serum AZPAb as an indicator for screening POF mice. By assessing the expression of serum AZPAb, morphological changes of ovarian tissue, levels of serum hormone (estradiol and gonadotropins) and apoptosis of GCs, the successful establishment of the autoimmune POF mice model was confirmed.

POF is biochemically characterized by low levels of estrogens and high levels of gonadotropins (hypergonadotropic amenorrhea) (Beck-Peccoz, et al., 2006). The results in the present study showed that the follicles of POF mice were significantly less sensitive to gonadotropins, the apoptotic GCs were significantly increased, the developing follicles were reduced, while the atretic follicles were



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increased obviously. These all suggest that ovarian dysfunction may have occurred in the successfully established POF mice model with pZP3 treatment.

GCs are the key cells in maintaining ovarian follicular development, secreting sex steroid hormones and growth factors that promote the oocyte growth. And they are in turn regulated by the gonadotrophins (Dumesic et al., 2015; Jankowska, 2017). Therefore, low estradiol level in POF mice resulted from the increased GCs apoptosis, where the feedback mechanism stimulated the pituitary gland to secrete gonadotropic hormones (high FSH and LH levels). Meanwhile, the excessive apoptosis of GCs was one of the important causes of follicular dysplasia in POF mice. Thus, ovarian failure occurred when the ovarian follicles of mice were exhausted.

It has been reported that ER stress was involved in the abnormal regulation of ovarian function (Wu et al., 2017). Caspase-12 is activated during ER stress. Studies have shown that the Caspase-12 deficiency mice were resistant to ER stress-induced apoptosis (Nakagawa et al., 2000). Caspase-12 is the key molecule of regulating ER stress-induced apoptosis. The activated Caspase-12 would enter cytoplasmic matrix from ER and activate Caspase-9. Next, Caspase-3 would be activated and eventually cell apoptosis caused (Hitomi et al., 2004). In our study, we found that the expression of Caspase-12 was significantly increased in ovarian tissues of POF mice. The results suggested that ER stress signaling pathway was

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involved in autoimmune induced POF mice. Studies have shown that the normal physiological function of GCs is dependent on the homeostatic maintenance of ER  $\text{Ca}^{2+}$  store (Sun et al., 2018). Therefore, in the present study, the GCs apoptosis of POF mice may be triggered by the ER stress response.

Once the ER state is disrupted, a large number of unfolded/misfolded proteins will aggregate. As the stress sensor, IRE1 $\alpha$  will separate from GRP78 and activate the ER stress signaling pathway (Walter and Ron, 2011; Stone and Lin, 2015). Upon activation, IRE1 $\alpha$  initiates the specific splicing of transcription factor X-box binding protein 1 (XBP1) mRNA (Calton et al., 2002; Yoshida et al., 2001). The spliced XBP1 mRNA encodes a functional protein XBP1s to regulate ER stress response genes such as GRP78, to resume cellular homeostasis by reducing unfolded/misfolded proteins (Gardner and Walter, 2011). In the present study, the data show the protein levels of IRE1 $\alpha$ , XBP1 and GRP78 were significantly increased and suggest that these proteins were involved in the ovarian dysfunction of POF mice. Furthermore, it is reported that excessive activation of IRE1 $\alpha$  could weaken the stress tolerance and induce cell apoptosis by activating downstream signaling molecule CHOP (Ron and Hubbard, 2008). As the most important mediator of ER stress-induced apoptosis, CHOP is able to increase the expression of apoptosis-induced substrate BIM, while decreasing expression of Bcl-2 (Puthalakath et al., 2007). These suggest that ER

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stress-related IRE1 $\alpha$  signaling pathway plays a central role in GCs apoptosis of ovaries in POF mice.

In the present study, we also evaluated the effect of hPMSCs transplantation on ovarian dysfunction of POF mice. The present results showed that hPMSCs transplantation significantly inhibited the excessive apoptosis of GCs in POF mice. And we also found that after transplantation of hPMSCs, the low estradiol level and high gonadotropins levels in POF mice were reversed, and the ovarian follicular dysplasia was improved. These indicate that hPMSCs transplantation is beneficial for the recovery of ovarian function of POF mice. Previous studies have shown that the hPMSCs transplantation could promote follicular development, and improve the ovarian reserve capacity (Zhang et al., 2018; Yin et al., 2018). These are consistent with our results. The findings strongly suggest that transplantation of hPMSCs is effective in treating ovarian disorders of POF mice. Next, we propose that hPMSCs transplantation attenuated GCs apoptosis via inhibiting the excessive activation of IRE1 $\alpha$  ER stress pathway. In the present study, following transplantation of hPMSCs, the protein expression of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12 were significantly inhibited in the ovaries of POF mice. Taken together, the results indicate that hPMSCs transplantation inhibited the activation of IRE1 $\alpha$ , attenuated the up-regulation of XBP1, GRP78 and Caspase-12 in POF mice, which subsequently result in the reduction of GCs

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apoptosis. These data supported our hypothesis that the excessive activation of IRE1 $\alpha$  signaling pathway might serve as a key mechanism of GCs apoptosis in pZP3-induced POF mice. And hPMSCs transplantation reduced the apoptosis of GCs in ovaries of POF mice by suppressing the activation of IRE1 $\alpha$  pathway.

## **5. Conclusions**

In summary, hPMSCs transplantation was helpful to improve the ovarian function and the follicular development of pZP3-induced autoimmune POF mice. The recovery of ovarian disorder may be related to the reduced apoptosis of GCs induced by ER stress-related IRE1 $\alpha$  signaling pathway. The findings also provided a potential therapeutic approach for the clinical treatment of patients with POF.

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## Conflict of interest

The authors declare that there are no conflicts of interest.

## References

- Badawy A, Sobh MA, Ahdy M, Abdelhafez MS (2017) Bone marrow mesenchymal stem cell repair of cyclophosphamide-induced ovarian insufficiency in a mouse model. *Int J Womens Health* 9: 441-447.
- Beck-Peccoz P, Persani L (2006) Premature ovarian failure. *Orphanet J Rare Dis* 1: 9.
- Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing

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---

the XBP-1 mRNA. *Nature* 415: 92-96.

Dumesic DA, Meldrum DR, Katz-Jaffe MG, Krisher RL, Schoolcraft WB (2015)

Oocyte environment: follicular fluid and cumulus cells are critical for oocyte health.

*Fertil Steril* 103: 303-316.

Fenton AJ (2015) Premature ovarian insufficiency: Pathogenesis and management. *J*

*Midlife Health* 6: 147-153.

Fu X, He Y, Wang X, Peng D, Chen X, Li X, Wang Q (2017) Overexpression of miR-21 in stem cells improves ovarian structure and function in rats with chemotherapy-induced ovarian damage by targeting PDCD4 and PTEN to inhibit granulosa cell apoptosis. *Stem Cell Res Ther* 8: 187.

Gao F, Zhang J, Wang X, Yang J, Chen D, Huff V, Liu YX (2014) Wt1 functions in ovarian follicle development by regulating granulosa cell differentiation. *Hum Mol*

---

Genet 23: 333-341.

Gardner BM, Walter P (2011) Unfolded proteins are Ire1-activating ligands that directly induce the unfolded protein response. *Science* 333: 1891-1894.

Hetz C, Chevet E, Oakes SA (2015) Proteostasis control by the unfolded protein response. *Nat Cell Biol* 17: 829-838.

Hetz C, Chevet E, Harding HP (2013) Targeting the unfolded protein response in disease. *Nat Rev Drug Discov* 12: 703-719.

Hewlett M, Mahalingaiah S (2015) Update on primary ovarian insufficiency. *Curr Opin Endocrinol Diabetes Obes* 22: 483-489.

Hitomi J, Katayama T, Taniguchi M, Honda A, Imaizumi K, Tohyama M (2004) Apoptosis induced by endoplasmic reticulum stress depends on activation of caspase-3 via caspase-12. *Neurosci Lett* 357: 127-130.

Hoek A, Schoemaker J, Drexhage HA (1997) Premature ovarian failure and ovarian autoimmunity. *Endocr Rev* 18: 107-134.

---

Jankowska K (2017) Premature ovarian failure. *Prz Menopauzalny* 16: 51-56.

Kuo FT, Bentsi-Barnes IK, Barlow GM, Pisarska MD (2011) Mutant Forkhead

L2

(FOXL2) proteins associated with premature ovarian failure (POF) dimerize with

wild-type FOXL2, leading to altered regulation of genes associated with

granulosa

cell differentiation. *Endocrinology* 152: 3917-3929.

Li J, Yu Q, Huang H, Deng W, Cao X, Adu-Frimpong M, Yu J, Xu X (2018)

Human chorionic plate-derived mesenchymal stem cells transplantation restores

ovarian function in a chemotherapy-induced mouse model of premature ovarian

failure. *Stem Cell Res Ther* 9: 81.

Luo AM, Garza KM, Hunt D, Tung KS (1993) Antigen mimicry in autoimmune

disease sharing of amino acid residues critical for pathogenic T cell activation. *J*

*Clin Invest* 92: 2117-2123.

Moore KA, Hollien J (2012) The unfolded protein response in secretory cell

function. *Annu Rev Genet* 46: 165-183.



---

Myers M, Britt KL, Wreford NG, Ebling FJ, Kerr JB (2004) Methods for quantifying follicular numbers within the mouse ovary. *Reproduction* 127: 569-580.

Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 403: 98-103.

Ostensen M, Brucato A, Carp H, Chambers C, Dolhain RJ, Doria A, Förger F, Gordon C, Hahn S, Khamashta M (2011) Pregnancy and reproduction in autoimmune rheumatic diseases. *Rheumatology (Oxford)* 50: 657-664.

Park JH, Hwang I, Hwang SH, Han H, Ha H (2012) Human umbilical cord blood-derived mesenchymal stem cells prevent diabetic renal injury through paracrine action. *Diabetes Res Clin Pract* 98: 465-473.

Parolini O, Alviano F, Bagnara GP, Bilic G, Bühring HJ, Evangelista M, Hennerbichler S, Liu B, Magatti M, Mao N, Miki T, Marongiu F, Nakajima H, Nikaido T, Portmann-Lanz CB, Sankar V, Soncini M, Stadler G, Surbek D, Takahashi TA, Redl H, Sakuragawa N, Wolbank S, Zeisberger S, Zisch A, Strom

---

SC (2008) Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. *Stem Cells* 26: 300-311.

Pedersen T, Peters H (1968) Proposal for a classification of oocytes and follicles in the mouse ovary. *J Reprod Fertil* 17: 555-557.

Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, Hughes PD, Michalak EM, McKimm-Breschkin J (2007) ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* 129: 1337-1349.

Ron D, Hubbard SR (2008) How IRE1 reacts to ER stress. *Cell* 132: 24-26.

Sano R, Reed JC (2013) ER stress-induced cell death mechanisms. *Biochim Biophys Acta* 1833: 3460-3470.

Silva CA, Yamakami LY, Aikawa NE, Araujo DB, Carvalho JF, Bonfá E (2014) Autoimmune primary ovarian insufficiency. *Autoimmun Rev* 13: 427-430.

Stone S, Lin W (2015) The unfolded protein response in multiple sclerosis. *Front Neurosci* 9: 264.

Sun Z, Zhang H, Wang X, Wang QC, Zhang C, Wang JQ, Wang YH, An CQ, Yang KY, Wang Y, Gao F, Guo C, Tang TS (2018) TMCO1 is essential for

---

ovarian follicle development by regulating ER  $\text{Ca}^{2+}$  store of granulosa cells. *Cell Death Differ* 25: 1686-1701.

Takamizawa S, Shibahara H, Shibayama T, Suzuki M (2007) Detection of antizona pellucida antibodies in the sera from premature ovarian failure patients by a highly specific test. *Fertil Steril* 88: 925-932.

Walter P, Ron D (2011) The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 334: 1081-1086.

Wu Y, Li Y, Liao X, Wang Z, Li R, Zou S, Jiang T, Zheng B, Duan P, Xiao J (2017) Diabetes induces abnormal ovarian function via triggering apoptosis of granulosa cells and suppressing ovarian angiogenesis. *Int J Biol Sci* 13: 1297-1308.

Yin N, Zhao W, Luo Q, Yuan W, Luan X, Zhang H (2018) Restoring ovarian function with human placenta-derived mesenchymal stem cells in autoimmune-induced premature ovarian failure mice mediated by Treg cells and associated cytokines. *Reprod Sci* 25: 1073-1082.

Yin N, Wang Y, Lu X, Liu R, Zhang L, Zhao W, Yuan W, Luo Q, Wu H, Luan X, Zhang H (2018) hPMSC transplantation restoring ovarian function in premature ovarian failure mice is associated with change of Th17/Tc17 and Th17/Treg cell ratios through the PI3K/Akt signal pathway. *Stem Cell Res Ther* 9: 37.

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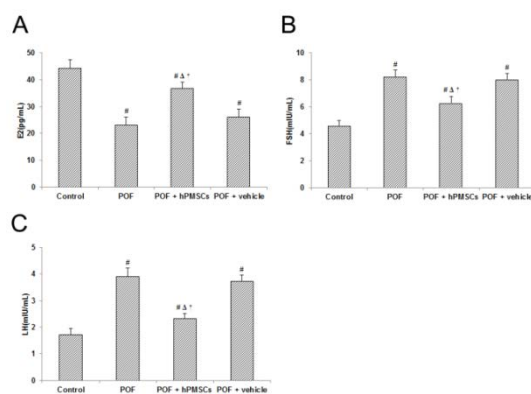
Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 107: 881-891.

Zhang H, Luo Q, Lu X, Yin N, Zhou D, Zhang L, Zhao W, Wang D, Du P, Hou Y, Zhang Y, Yuan W (2018) Effects of hPMSCs on granulosa cell apoptosis and AMH expression and their role in the restoration of ovary function in premature ovarian failure mice. *Stem Cell Res Ther* 9: 20.

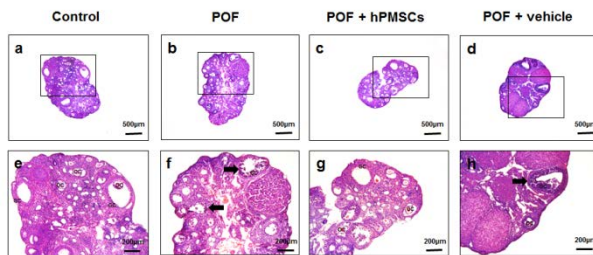
## Figures

### Figure 1. The comparison of serum hormones secretion in each group mice.

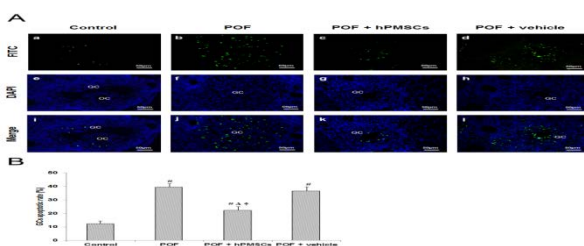
The levels of hormones were measured by ELISA assay. A, Estradiol (E<sub>2</sub>). B, Follicle stimulating hormone (FSH). C, Luteinizing hormone (LH). Data are presented as the means  $\pm$  SD.  $n = 10$  for each group. Tukey's test: # $P < 0.01$  compared with Control group;  $\Delta P < 0.01$  compared with POF group;  $\dagger P < 0.01$  compared with POF + vehicle group.



**Figure 2. Representative images of H&E staining on ovarian tissues from mice in each group.** Pictures e, f, g and h were magnifications of the rectangle in photos a, b, c and d, respectively. In a, b, c and d, scale bar = 500  $\mu\text{m}$ ; in e, f, g and h, scale bar = 200  $\mu\text{m}$ . Thick black arrow refers to the atretic follicle. GC: granulosa cell; OC: oocyte.



**Figure 3. Apoptosis of GCs in ovaries was measured by TUNEL staining. A:** Representative images of TUNEL staining shown apoptotic GCs in each group. The TUNEL-positive apoptotic cells were shown as green fluorescence with fluorescein isothiocyanate (FITC) staining. The nuclei (blue) were stained with DAPI. Scale bar = 50  $\mu\text{m}$ . B: the percentage of TUNEL-positive GCs in each group. Data are presented as means  $\pm$  SD.  $n = 5$  for each group. Tukey's test:  $\#P < 0.01$  compared with Control group;  $\Delta P < 0.01$  compared with POF group;  $\dagger P < 0.01$  compared with POF + vehicle group. GC: granulosa cell; OC: oocyte.



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**Figure 4. Expression of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12 in ovaries**

**analyzed by immunohistochemical staining.** A, Representative

immunohistochemical images of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12 in each

group. Brown staining represents the positive signal of IRE1 $\alpha$ , XBP1, GRP78 and

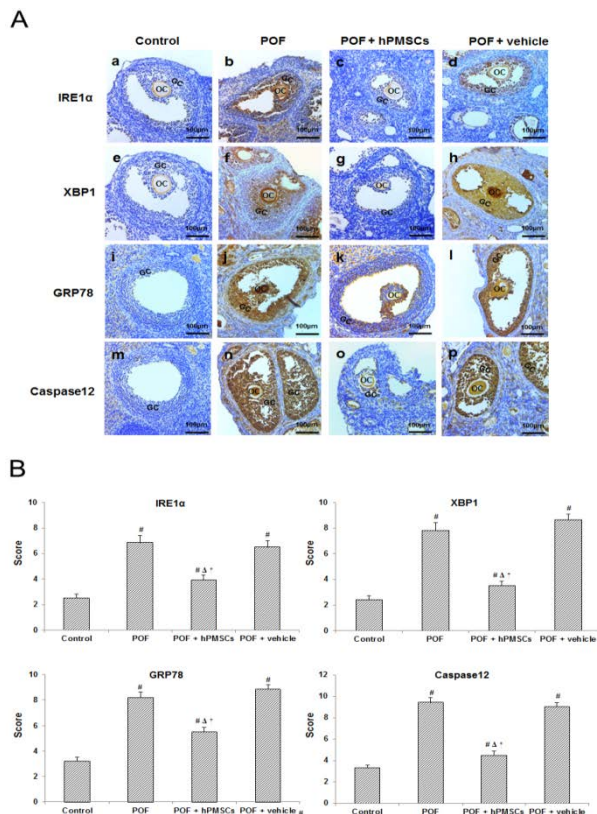
Caspase-12. Cell nucleus stained blue. Scale bar = 100  $\mu$ m. GC: granulosa cell;

OC: oocyte. B, Optical density values of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12

positive products in each group. Data are presented as the means  $\pm$  SD.  $n = 5$  for

each group. Tukey's test:  $^{\#}P < 0.01$  compared with Control group;  $^{\Delta}P < 0.01$

compared with POF group;  $^{\dagger}P < 0.01$  compared with POF + vehicle group.



**Figure 5. The protein expression of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12**

**in ovaries analyzed by Western blotting.** A, Representative immunoblots of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12 proteins in each group.  $\beta$ -actin protein expression was served as loading control. B, The quantitation of IRE1 $\alpha$ , XBP1, GRP78 and Caspase-12 protein expression in each group. Data are presented as the means  $\pm$  SD.  $n = 9$  for Control group;  $n = 15$  for POF group;  $n = 15$  for POF + hPMSCs group;  $n = 15$  for POF + vehicle group. Tukey's test: # $P < 0.01$  compared with Control group;  $\Delta P < 0.01$  compared with POF group;  $\dagger P < 0.01$  compared with POF + vehicle group.

