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Effects of mesenchymal stem cells on ovarian sirtuin 1 and caspase-3 in premature ovarian insufficiency mice

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ABSTRACT: Human umbilical cord mesenchymal stem cells (HUCMSCs) can promote angiogenesis and reduce apoptosis, but their effects in premature ovarian insufficiency (POI) remain unclear. We found that HUCMSCs could improve ovarian function in POI mice through sirtuin 1 (SIRT1)/caspase-3. Female C57BL/6 mice ($n = 45$) were randomly divided into 3 groups: the POI and HUCMSC groups receiving cyclophosphamide for 14 days to induce POI and the control group receiving normal saline injections. HUCMSCs were injected into the tail veins of the mice in the HUCMSC group on days 14 and 28. We analyzed the mouse body and ovarian weight, ovarian structure, number of ovarian follicles, estrous cycles, serum hormone levels, ovarian SIRT1 and caspase-3 expression, and granulosa-cell apoptosis. The POI group showed decreased body weight, ovarian weight, number of follicles, and SIRT1 expression as well as impaired ovarian hormones, and increased caspase-3 and granulosa-cell apoptosis. Body weight, ovarian weight, disturbed estrous cycle, number of follicles, and hormone levels were improved after the HUCMSC intervention, while the ovarian SIRT1 level was increased, and caspase-3 and granulosa-cell apoptosis were decreased. HUCMSCs promote SIRT1 expression and downregulate caspase-3, thereby reducing ovarian granulosa-cell apoptosis and improving ovarian function in POI mice.

KEYWORDS: apoptosis, human umbilical cord mesenchymal stem cells, premature ovarian insufficiency, SIRT1, caspase-3

INTRODUCTION

Premature ovarian insufficiency (POI) refers to the loss of ovarian function in women before the age of 40 years. The incidence rate of POI is approximately 1%; however, in recent years, the incidence of POI has been gradually increasing owing to lifestyle and environmental changes [1]. The clinical characteristics of POI include menstrual disorders, elevated follicle-stimulating hormone (FSH) level, decreased estrogen (E2) level, and ovarian follicle dysfunction or follicle depletion [2]. Infertility, menstrual disorders, and low estrogen symptoms caused by decreased ovarian function have long been a hot spot of research and discussion. Studies have found that POI is associated with a natural pregnancy rate of only 1.5–4.4%, and as more and more women are being diagnosed with POI before childbearing, POI seriously affects the physiological and reproductive health of women of childbearing age [3]. Currently, the treatment of POI mainly includes estrogen-progesterone supplementation therapy, ovulation promotion, immunization, and traditional Chinese medicine [4]; however, these methods cannot effectively restore ovarian function and fertility.

Stem cells are primitive cells with multi-directional differentiation potential and self-renewal capacity and can be used to restore various tissue functions [5]. Human umbilical cord mesenchymal stem cells (HUCMSCs) are convenient to obtain and easy to preserve. HUCMSCs have been used for the treatment of hematological, neurological, and other diseases [6]. However, these cells have rarely been applied in the treatment of POI, and their potential mechanism in ovarian function is unclear.

Sirtuin 1 (SIRT1) is a nicotinamide adenosine dinucleotide-dependent histone deacetylase (HDAC) that plays a role in inhibiting oocyte aging, improving ovarian reserve function, and prolonging ovarian life span [7]. One study [8] found that SIRT1 can inhibit the transcription factor forkhead box protein O1 (FoxO1) in ovarian granulosa cells, thereby inhibiting granulosa-cell apoptosis due to oxidative damage. However, it is unclear whether HUCMSCs can improve ovarian function in mice with POI by reducing the apoptosis of ovarian granulosa cells via SIRT1. Therefore, in this study, we established a mouse model of POI to investigate the role and mechanism of HUCMSCs on

ovarian function in POI mice.

MATERIALS AND METHODS

Laboratory animals and main reagent drugs

We acquired 7-week-old healthy female C57BL/6 mice from Hunan SJA Laboratory Animal Co. Ltd., China ($n = 45$; Animal Use License No. SYXK Xiang 2022-0002). Cyclophosphamide was provided by Solarbio Company (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits for anti-Mullerian hormone (AMH), FSH, and E2 were purchased from Sangon Biotech Co. Ltd. (Shanghai, China). SIRT1 and cysteinyl aspartate specific proteinase-3 (caspase-3) primers were designed and synthesized by Changsha Dexin Biotechnology Co. (Hunan, China). Caspase-3 and SIRT1 anti-mouse primary antibodies were purchased from CST Company (Boston, MA, USA), and the Colorimetric TUNEL Apoptosis Assay Kit was purchased from Beyotime Biotech Inc. (Shanghai, China). This experiment was approved by the ethics committee of Loudi Hospital, University of South China (No. 2022-ethical review (scientific research)-050). The Guidelines for the Care and Use of Laboratory Animals were followed.

Isolation, culture, and characterization of HUCMSCs

We collected the umbilical cords of healthy full-term infants born via caesarean section in Loudi Hospital of Nanhua University. The umbilical cord samples were washed with phosphate-buffered saline (PBS), cut into pieces smaller than 1 mm^2, and incubated overnight with 4 ml of 0.1% type II collagenase at 4 °C. The next day, the PBS was rinsed off, and the samples were washed twice with complete medium, poured into dishes, and incubated with 3 ml of the medium on an undigested tissue block. After the tissue was completely digested, 4 ml of culture medium was added to continue the culture. After the cell number increased significantly, the cells were transferred to two 50-ml centrifuge tubes. Next, 50 ml of PBS was added to the samples and mixed well. The samples were centrifuged at 2,500 rpm for 6 min and then resuspended in 6 ml of medium and again centrifuged at 1,000 rpm for 6 min. The cells obtained were spread in a T25 flask and incubated with 5 ml of complete medium. The culture medium was changed daily. When the cells were grown to 80% confluence, they were passaged. Cells at passage 3 were analyzed for the expression of HUCMSC surface marker molecules, and the HUCMSCs identified were collected. We prepared a single-cell suspension of HUCMSCs at a concentration of 2.5×10^6 cells/ml per tube. The procedure of human umbilical cord collection was approved by the Ministry of Science and Technology, PRC (No. 2022SLCJ2020). All the enrolled pregnant women signed informed consent forms for the collection of human umbilical cord tissue.

Animal grouping, modeling, and interventions

Female C57BL/6 mice with a normal estrous cycle were randomly divided into a POI group, an HUCMSC group, and a control group ($n = 15$ per group). The mice in the POI and HUCMSC groups received cyclophosphamide (70 mg/kg/d) via continuous intraperitoneal injection for 14 days, while the control mice received daily isovolume normal saline gavages during the modeling period. On days 14 and 28, the mice in the HUCMSC group were injected with the 2.5×10^6 HUCMSCs, while the mice in the POI and control groups were injected with an equal volume of normal saline during the treatment period. The animal experiments were ethically approved by the Laboratory Animal Ethical Review Committee of Hunan Yuanhe Biotechnology Co. Ltd. (no. HNYH2022016).

Animal specimen processing

From day 9 of the modeling period and day 9 of the last HUCMSC intervention, cells shed from the vagina were collected from each group of mice for 5 days. On day 14 after the last HUCMSC intervention, the mice were anesthetized, and their orbital blood was collected. The blood sample was allowed to solidify at room temperature and then centrifuged at 1,200 rpm at 4 °C for approximately 20 min. The supernatant was collected, and the resultant serum was frozen at −80 °C. The ovaries were isolated and weighed. One ovary from each mouse was fixed in 4% paraformaldehyde for hematoxylin and eosin staining, immunohistochemistry, and TUNEL assays, while the other ovary was rapidly stored in liquid nitrogen for reverse transcription and quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis.

Measurements of hormone levels

The murine serum AMH, FSH, and E2 levels were determined using ELISA, which was performed strictly according to the manufacturers' instructions.

Ovarian histopathology and number of follicles

Ovarian tissue samples were embedded in paraffin, sectioned at 5-μm intervals, dewaxed, hydrated, stained with hematoxylin and eosin, dehydrated, made transparent, sealed, and examined under a microscope. The number of follicles was counted in a blind fashion.

Immunohistochemistry

Paraffin-embedded ovarian tissue sections were dewaxed, dehydrated, and placed in an antigen-repair solution. The endogenous enzymes were inactivated, and the sections were washed with PBS and blocked with goat serum. The liquids were drained, and

the sections were incubated with the SIRT1 (1:100) and caspase-3 (1:300) primary antibodies at 4 °C overnight. Next, the sections were thawed at room temperature and washed with PBS. The bound antibodies were detected via incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody for 30 min. The sections were washed again with PBS, stained blue with diaminobenzidine (DAB), dehydrated, clarified, and wet-sealed.

Western blot analysis

Proteins were extracted from ovarian tissue samples, subjected to gel electrophoresis, and transferred onto polyvinylidene difluoride membranes. Then, the membranes were incubated with the primary antibodies against SIRT1 and caspase-3 at 4 °C overnight. The bound antibodies were detected using HRP-conjugated secondary antibodies. Specific protein bands were visualized using enhanced chemiluminescence and quantified using Quantity One software. GAPDH was used as the internal control.

RT-qPCR assay

Total RNA was extracted from an equal amount of ovarian tissue samples using TRIZOL. After qualification and quantification, the RNA samples were reverse-transcribed into cDNA. The relative levels of SIRT1 and caspase-3 mRNA to the control GAPDH mRNA transcripts in individual ovarian tissue samples were quantified using RT-qPCR with specific primers (Table S1). The PCR amplification conditions were as follows: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 60 °C for 1 min, and 95 °C for 15 s. The obtained data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Apoptosis detection using TUNEL assay

Paraffin-embedded ovarian tissue sections were dewaxed, hydrated, and washed, and a biotin labeling solution was dripped onto the sections. The sections were incubated at 37 °C for 60 min and then washed with PBS. Next, 0.2 ml of the labeled reaction termination solution was added, and the sections were further incubated at room temperature for 10 min. After being washed, the sections were treated with the streptavidin-HRP working solution and incubated at room temperature for another 30 min. Finally, the sections were visualized with DAB and then counterstained with hematoxylin. Apoptotic cells in the tissue sections appeared brown under a microscope. The percentage of positively stained cells among every 500 cells was defined as the apoptosis index.

Statistical analysis

Data were analyzed using SPSS version 19.0 and expressed as mean ± standard deviation ($\bar{x} \pm s$). Analysis of variance followed by the least significance difference or Tamhane T2 test was used to compare differences in the data between 2 groups. $p < 0.05$ was considered statistically significant.

Table 1 Comparison of mouse body weight and ovarian weight in each group.

Group	Body weight (g)	Ovarian weight (mg)
Control	21.20 ± 0.86	6.78 ± 0.57
POI	16.08 ± 0.48*	2.96 ± 0.42*
HUCMSC	17.52 ± 0.93*$^{\Delta}$	3.94 ± 0.40*$^{\Delta}$

* $p < 0.05$ vs. the control group; $^{\Delta}$ $p < 0.05$ vs. the POI group ($n = 15$ per group). POI, premature ovarian insufficiency; HUCMSCs, human umbilical cord mesenchymal stem cells.

RESULTS

Characterization of HUCMSCs

To confirm whether the cells derived from the fetal umbilical cords have mesenchymal stem cell properties, we performed cell differentiation and flow cytometric analyses. The cells could be induced to develop into the following lineages: adipogenesis, osteogenesis, and chondrogenic differentiation (Fig. 1). The expression rates of CD90, CD44, CD105, and CD73 in the HUCMSCs were 85.61%, 76.75%, 84.22%, and 85.60%, respectively.

Comparison of mouse body weight and ovarian weight in each group

Compared with the control mice, the mice in the POI group showed significantly reduced body weight and ovarian weight ($p < 0.05$). Compared with the POI group, the HUCMSC-group mice showed significantly increased body weight and ovarian weight ($p < 0.05$, Table 1).

Comparison of estrous cycles of mice in each group

The estrous cycle of normal mice is generally 4–5 days, which is divided into preestrous, estrum, anestrum, and metestrous (Fig. 2). Estrous cycle disorder was observed in the model mice. After the HUCMSC intervention, the disturbed estrous cycle was improved, and most mice could recover their regular estrous cycle.

Comparison of serum AMH, FSH, and E2 levels in each group

Compared with the control group, the POI group showed decreased serum AMH and E2 levels and increased FSH level ($p < 0.05$). Compared with the POI group, the HUCMSC group showed increased serum AMH and E2 levels and decreased FSH level ($p < 0.05$, Table 2).

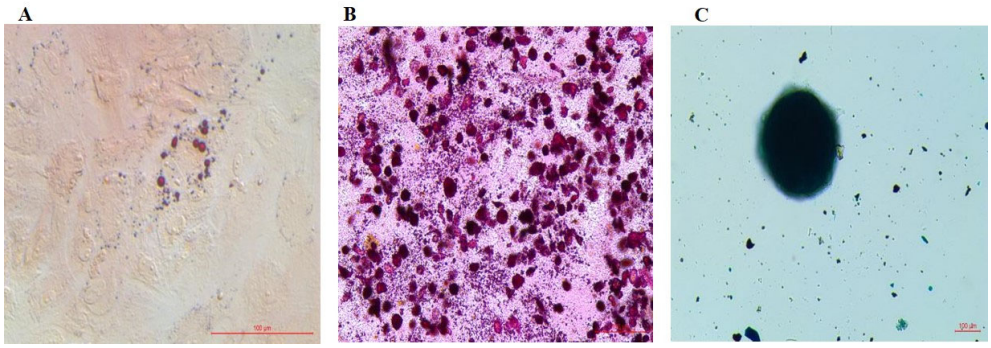

Fig. 1 Characterization of human umbilical cord mesenchymal stem cells. (A)–(C): Differentiated adipocytes, osteoblasts, and chondroblasts, respectively.

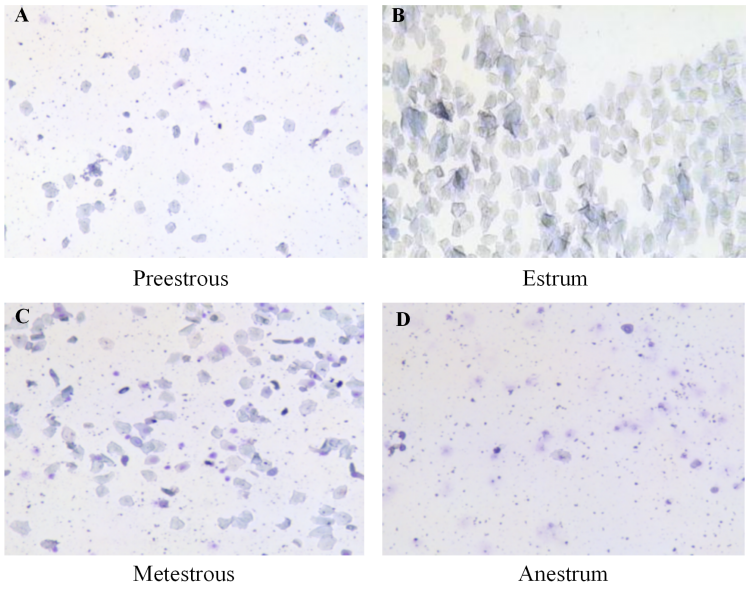

Fig. 2 Vaginal discharge smears of the mice. (A) Preestrous: almost all cells being nucleated keratinized epithelial cells. (B) Estrum: almost all cells being nucleus-free keratinized epithelial cells. (C) Metestrous: nucleated cells, anucleated cells, and leukocytes present together. (D) Anestrum: large numbers of leukocytes seen.

Comparison of ovarian histomorphology and number of follicles in each group

Upon visual inspection, the ovarian volume was smaller in the POI group than that in the control group; the ovarian volume increased after the administration of HUCMSCs (Fig. 3A). Microscopic examination of hematoxylin and eosin-stained ovarian tissue sections revealed well-developed mature follicles and orderly granulosa cells in the control group. In POI group, the number of follicles was decreased relative to the control group ($p < 0.05$), and the arrangement of the granulosa cells was disordered. In the HUCMSC group, the number of follicles was increased relative to the POI group ($p < 0.05$), and the arrangement of the granulosa cells was orderly (Fig. 3B,C).

Table 2 Comparison of serum AMH, FSH, and E2 levels in each group.

Group	AMH (ng/ml)	FSH (IU/l)	E2 (pg/ml)
Control	1.39 ± 0.19	8.08 ± 0.87	30.99 ± 0.33
POI	$0.77 \pm 0.18^{*}$	$13.24 \pm 0.72^{*}$	$22.43 \pm 0.52^{*}$
HUCMSC	$1.12 \pm 0.09^{*\Delta}$	$12.34 \pm 0.78^{*\Delta}$	$23.83 \pm 0.43^{*\Delta}$

* $p < 0.05$ vs. the control group; $^{\Delta}$ $p < 0.05$ vs. the POI group ($n = 15$ per group). AMH, anti-Mullerian hormone; FSH, follicle-stimulating hormone; E2, estrogen; POI, premature ovarian insufficiency; HUCMSC, human umbilical cord mesenchymal stem cell.

SIRT1 and caspase-3 expression

After immunohistochemical staining, cells that were positive for the SIRT1 and caspase-3 proteins appeared tan. SIRT1 was mainly expressed in the granulosa

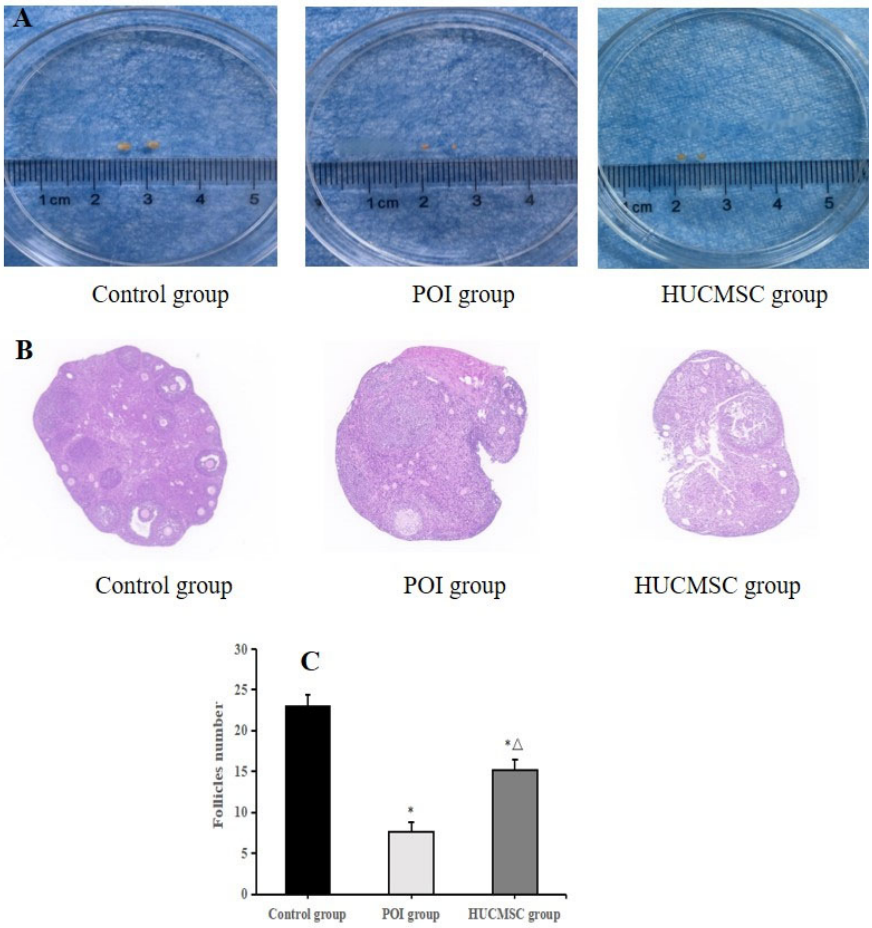

Fig. 3 Treatment with HUCMSCs preserving the ovarian mass and limiting follicle degeneration in mice following POI induction. (A) Visual observation of ovarian mass in the different groups of mice. (B) Hematoxylin and eosin staining of ovarian tissues (× 200). (C) Comparison of the number of follicles in each group. * $p < 0.05$ vs. the control group;$^{\triangle}$ $p < 0.05$ vs.$ the POI group ($n = 15$ per group). HUCMSCs, human umbilical cord mesenchymal stem cells; POI, premature ovarian insufficiency.

cells in the control group, and caspase-3 was mainly expressed in the granulosa cells in the POI group (Fig. 4).

Effects of HUCMSCs on SIRT1 and caspase-3 expression in ovarian granulosa cells

Compared with the control group, the POI group exhibited decreased SIRT1 expression, increased caspase-3 expression, and increased apoptosis in the ovarian granulosa cells. After the HUCMSC intervention, SIRT1 expression increased, caspase-3 expression decreased, and the apoptosis rate decreased among the granulosa cells ($p < 0.05$, Fig. 5A–E).

DISCUSSION

Currently, the treatment of POI mainly consists of hormone supplementation therapy. Although this treatment can relieve many of the symptoms caused by low estrogen, it cannot meet the fertility needs of patients [2]. Stem cells have the potential to perform the functions of various tissues and organs and to regenerate parts of the human body; these versatile cells can, under certain conditions, differentiate into a variety of functional cells that can be used to treat various diseases [9]. According to their developmental potential, stem cells can be divided into (1) totipotent stem cells (fertilized egg), (2) pluripotent stem cells (e.g., embryonic stem cells), and (3) unipotent stem cells (e.g., neural stem cells and hematopoietic stem cells) [9, 10]. Embryonic stem cell-derived HUCMSCs are considered an ideal stem cell source for treating clinical diseases due to their advantages of easy access and strong proliferation and differentiation ability [11]. In the studies on the migration and distribution of HUCMSCs in tissue, fluorescent labeled HUCMSCs were injected into the tail veins of mice

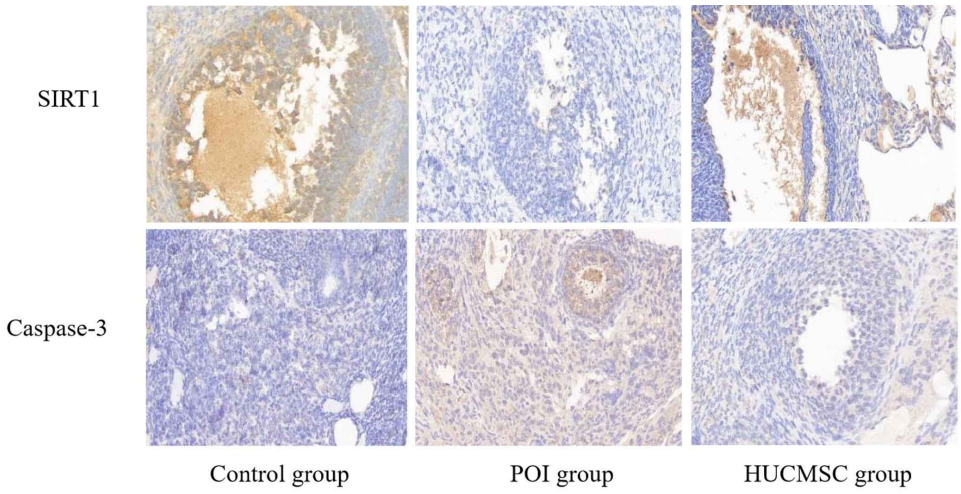

Fig. 4 SIRT1 and caspase-3 expression in mouse ovaries (×400). SIRT1, sirtuin 1; POI, premature ovarian insufficiency; HUCMSCs, human umbilical cord mesenchymal stem cells.

and were subsequently found in the heart, spleen, lungs, kidneys, ovaries, and other organs; at 24 h after administration, the fluorescence intensity in ovarian tissue was enhanced, and the labeled HUCMSCs in ovarian granulosa cells remained strongly fluorescent until 72 h [12, 13], proving that HUCMSCs can migrate to the ovary. It has also been found that AMH can regulate the growth and development of ovarian follicles, and AMH levels are highly sensitive and specific [14] for the assessment of ovarian reserve function. Furthermore, AMH and E2 have a negative feedback effect on follicle development, which can affect the FSH level [15]. In this study, we found that the E2 and AMH levels were decreased, the number of follicles was decreased, and the FSH level was increased in the POI mice relative to the control mice. After the HUCMSC intervention, the AMH and E2 levels increased, the FSH level decreased, and the estrous cycle of the mice was restored to the normal pattern. Additionally, the body weight, ovarian weight, and number of follicles also increased. The above findings may be explained as follows: as E2 is produced by the granulosa cells in the follicles, the increase in E2 in the POI mice after the HUCMSC intervention promoted negative feedback from the pituitary, which reduced FSH level. AMH is also secreted by the granulosa cells, which can inhibit the start of primordial follicle growth, and as the number of ovarian primordial follicles increases, the serum AMH concentration also increases. It is speculated that HUCMSCs can migrate to the granulosa cells and improve ovarian function in POI mice, but the specific mechanism is currently unclear.

Granulosa cells are the main functional cells that synthesize and secrete E2 and AMH and regulate follicle growth, development, and maturation in an autocrine and paracrine manner [16]. Granulosa cell apoptosis is a normal physiological activity that is required for continuous ovarian development and maintaining a stable microenvironment and is closely related to other physiological processes such as follicular development, follicular atresia, and the menstrual cycle. However, excessive apoptosis of the granulosa cells may reduce the number of follicles, impair ovarian function, and lead to the occurrence of POI [17, 18].

The SIRT family consists of highly conserved HDACs, which are important in gene silencing, lifespan extension, and anti-aging processes [19]. There are 7 different SIRT members in mammals (SIRT1-SIRT7), among which SIRT1, a class III HDAC, was found in humans in 1999. SIRT1 can exist in various somatic cells and germ cells and participates in various physiological processes such as cell proliferation, differentiation, and apoptosis [20]. Gao et al [21] found that in rats with interstitial cystitis, the intrathecal injection of HUCMSCs could reduce neuroinflammation and oxidative stress through the Sirt1/Nrf2/HO-1 pathway. Microcurrent-stimulated HUCMSCs have been reported to inhibit apoptosis and subsequently exert neuroprotective effects through the lncRNA-MALAT1/miR22-3p/SIRT1/AMPK axis [22]. These reports show that HUCMSCs can regulate SIRT1 expression in various diseases. Guo et al [23] found that after SIRT1 knockdown, the expression of genes related to estrogen synthesis such as CYP17A1, NR5A1, STAR, and functional receptors was decreased, which affected ovarian reserve function and follicular development. SIRT1 can also inhibit the activity of tumor-associated protein 53 (p53) via deacetylation of the C terminal Lys382 of p53 and thus reduce the apoptosis and atresia of follicles and maintain

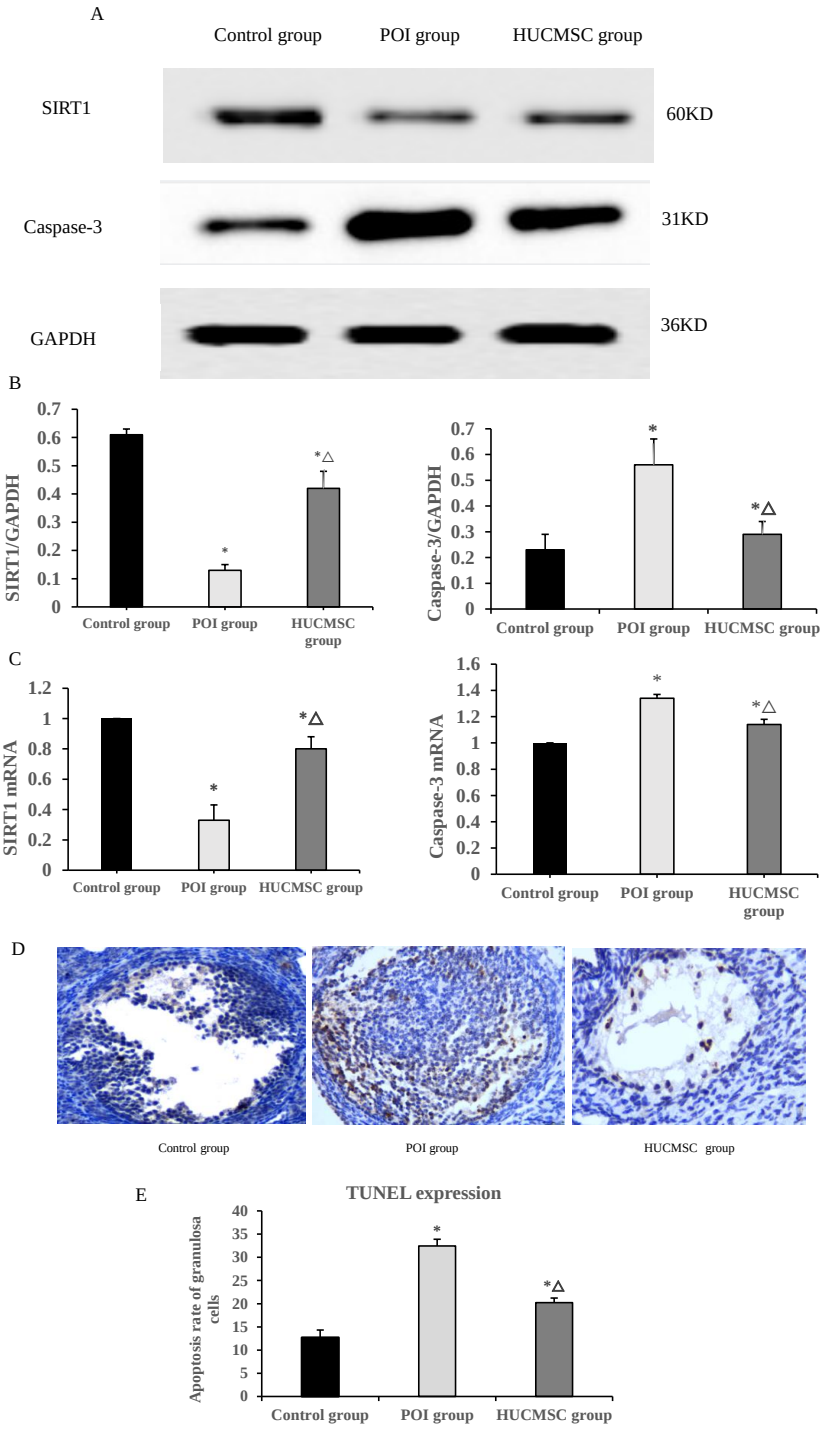

Fig. 5 Comparison of apoptosis of SIRT1, caspase-3, and ovarian granulosa cells in each mouse group. A. Western blot results of related proteins in each mouse group. B. Protein expression levels of SIRT1 and caspase-3 in each group. C. RT-qPCR expression levels of SIRT1 and caspase-3 in each group. D. TUNEL images of ovarian granulosa cells in each mouse group ($\times$400). E. Comparison of the apoptosis rate of ovarian granulosa cells in each mouse group. SIRT1, sirtuin 1; POI, premature ovarian insufficiency; HUCMSCs, human umbilical cord mesenchymal stem cells; RT-qPCR, quantitative reverse transcription polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

ovarian reserve [24]. In this study, SIRT1 was highly expressed in normal ovarian granulosa cells; SIRT1 expression was decreased in mice with POI induced by cyclophosphamide, and SIRT1 expression was enhanced after the HUCMSC intervention. The TUNEL assay also showed that after SIRT1 expression was increased following the HUCMSC treatment, the apoptosis of ovarian granulosa cells was decreased. We speculate that the promotion of SIRT1 expression by HUCMSCs may reduce the apoptosis of granulosa cells and improve ovarian function in POI mice. HUCMSCs can reverse hypoxia-induced apoptosis in β cells by inhibiting endoplasmic reticulum stress and reducing caspase-3 levels [25]. Other studies have found that SIRT1 in the mitochondrial pathway can inhibit the expression of p53, which reduces the expression of the apoptosis execution factor caspase-3 and inhibits the apoptosis of granulosa cells [26, 27]. In this study, we found that the ovarian caspase-3 protein and mRNA expressions were higher in the POI mice than those in the control mice, and that the HUCMSC intervention could promote SIRT1 expression and reduce the caspase-3 level.

CONCLUSION

This study found that HUCMSCs could increase the number of ovarian follicles, elevate the serum AMH and E2 levels, reduce the serum FSH level, and improve ovarian function in mice with POI. The underlying mechanism may be that HUCMSCs promote SIRT1 expression, which then downregulates caspase-3 expression, thereby reducing the apoptosis of ovarian granulosa cells and improving ovarian function in POI mice to some extent. These findings provide an experimental basis for the development of stem cell therapies for POI.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2024.093. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Table S1 PCR primer sequences.

Gene	Sequence (5′–3′)
SIRT1	F: GGA GCA GAT TAG TAA GCG GCT TG
	R: GTT ACT GCC ACA GGA ACT AGA GG
Caspase-3	F: GTC CCA CTG TCT GTC TCA
	R: GAA TGT CAT CTC GCT CTG
GAPDH	F: CGT GTT CCT ACC CCC AAT GT
	R: TGT CAT CAT ACT TGG CAG GTT TCT

PCR, polymerase chain reaction; SIRT1, sirtuin 1; GADPH, glyceraldehyde 3-phosphate dehydrogenase.