Effects of umbilical cord mesenchymal stem cells on expression of CYR61, FSH, and AMH in mice with premature ovarian failure

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ABSTRACT

This study aimed to investigate the effects of umbilical cord mesenchymal stem cells on the expression of CYR61, FSH and AMH in mice with premature ovarian failure. For this purpose, thirty SPF female SD mice were selected as the research object, 10 of which were control group, namely group α, and 20 mice with premature ovarian failure model were established by cyclophosphamide. The mice were divided into the model group, namely the β group and the umbilical cord mesenchymal stem cell transplantation group (γ group), with 10 mice in each group. ELSA method was used to determine the levels of follicle-stimulating hormone (FSH), Luteinizing hormone (LH), estradiol (Estradiol) in serum. The changes of E2, Antimullerian hormone (AMH) and cysteine-rich protein 61 in ovarian tissues were determined by the protein imprinting method. Connective tissue growth factor (CTGF) and caspase-3 protein expression. Results showed that in fertility rate, γ group > α group > β group, the difference was statistically significant (P<0.05), in litter size, α group > γ group > β group, the difference was statistically significant (P<0.05). The levels of serum E2 and AMH in α group > γ group > β group, and the levels of serum FSH and LH in β group > γ group > α group were statistically significant (P<0.05). The growth follicles were α group > γ group > β group, and the atresia follicles were β group > γ group > α group, and there was a statistical difference among all groups (P<0.05). There was no difference in luteal number among the three groups (P>0.05). In terms of CYR61 and CTGF protein expression, α group > γ group > β group, and in terms of caspase-3, β group > γ group > α group had statistical significance (P<0.05). It is concluded that intervention with umbilical cord mesenchymal stem cells can significantly improve the expression levels of CYR61 and AMH, reduce the level of FSH, promote cell survival, improve the reproductive quality of mice, and restore the physiological function of the ovary. It is feasible to treat premature ovarian failure with umbilical cord mesenchymal stem cells.

Introduction

Premature ovarian failure (POF) refers to the reduction and disappearance of ovarian follicles in women under 40 years old, resulting in irregular menstruation, amenorrhea and atrophy of sexual organs, which is a common clinical disease in obstetrics and gynecology, and also the main cause of female infertility today (1). Causes of premature ovarian failure may be autoimmune damage, mental stimulation, abnormal metabolism, smoking, drinking and another adverse lifestyle. However, up to now, the etiology and pathogenesis of most patients with premature ovarian failure have not been clear (2). At present, the treatment of premature ovarian failure can be restored through hormone therapy and immunotherapy. However, the treatment effect of the above methods is temporary and cannot effectively promote the regeneration and repair of ovarian tissue. Therefore, the current research focus in premature ovarian failure is how to restore the patient's ovarian function. Mesenchymal stem cells are adult stem cells from mesoderm with self-renewal, proliferation and multidirectional differentiation, which are widely used in tissue engineering and other aspects (3). Nowadays, there are many studies on bone marrow-derived

mesenchymal stem cells, but they are affected by age and may be contaminated by viruses and bacteria (4, 5). However, the number of mesenchymal stem cells from the amniotic fluid is limited, and umbilical cord blood mesenchymal stem cells have a certain degree of immunogenicity (6). Umbilical cord mesenchymal stem cells derived from neonatal umbilical cord tissue have no impact on the donor and low immunogenicity, and the infection rate of viruses and pathogenic microorganisms is lower than that of bone marrow mesenchymal stem cells, which can be passed for many times in vitro and maintain normal functions (7). Therefore, POF treatment has a good prospect. In this study, the expression of CYR61, FSH and AMH in mice model of premature ovarian failure after umbilical cord mesenchymal stem cells treatment was analyzed, and the therapeutic effect and mechanism of umbilical cord mesenchymal stem cells on premature ovarian failure were analyzed. The results are as follows.

Materials and methods
Experimental animals
There were 30 SPF BALB/ C female mice in the normal estrous cycle, weighing 18.0g to 22.2g, aged 7 to 8 weeks. Mice were uniformly fed in the animal experiment center of our hospital, with a room temperature of 22±2 °C, uninterrupted lighting at 12h: 12h day and night, and free drinking water. This study has been reviewed by the ethics committee of our hospital, and the experimental animals conform to the 3R principle.

Reagents and instruments
Cyclophosphamide for injection (Jiangsu Hengrui Pharmaceutical Co., LTD.); DMEM culture medium, fetal bovine serum (Cymofield); ELSA Detection Kit (Huamei Biological); Total protein extraction kit, BCA protein quantitative kit, gel protein luminescence system (Parker biology); Rabbit monoclonal antibody against human CYR61, CTGF, Caspase-3 (Abcam China).

Model preparation
Model mice were given one-time intraperitoneal injection of cyclophosphamide 120 mg/kg+ baixioan 30 mg/kg. The cyclophosphamide injection was prepared with normal saline as 20mg/ mL water-soluble injection, and DMSO was prepared as 66.7 mg/kg fat-soluble

injection. The control group was intraperitoneally injected with the same volume of DMSO solution.

Culture and identification of umbilical cord mesenchymal stem cells
After being rinsed with PBS solution, the umbilical cord, venous blood residue and blood vessels were removed. The umbilical cord, venous blood residue and blood vessels were cut into pieces with medical scissors with a size of 1-2mm3. Then the cut tissue was placed on the petri dish and cultured in an incubator with 37℃ and 5%CO2. The culture was continued as a single cell. Then trypsin digestion, routine cell passage.

Grouping of animals
In this study, 30 mice were divided into 3 groups, namely α, β and γ group, α group was normal control. Mice in β group were mice with premature ovarian failure, mice in γ group were mice with premature ovarian failure by tail vein injection of 1ml umbilical cord mesenchymal stem cell suspension, the cell volume was about $1×106$, and mice in β group and the two groups were given an equal dose of normal saline injection.

Detection of human umbilical cord mesenchymal stem cell surface antigen by flow cytometry
F5 generation cells were identified by flow cytometry. The adherent cells were digested by conventional trypsin and suspended at a density of $1×107$ to prepare cell suspension. Mouse anti-human CD105, CD73, CD34 and CD45 monoclonal antibodies were added, respectively. After incubation at room temperature for 30min, the expression of CD105, CD73, CD34 and CD45 antibodies was determined by upflow cytometry.

General situation analysis of mice
On day 15, mice were raised in cages with male and female mice at 2:1, and the birth rate, litter size and litter quality of mice were observed.

Determination of serum hormone levels
After 15 days of umbilical cord mesenchymal stem cell transplantation, blood was taken from the orbit of mice in all three groups and centrifuged at 3000r/min for 15min, but a supernatant was obtained. Enzyme-linked immunoassay was used to determine follicle-stimulating hormone (FSH), Luteinizing hormone (LH),

estradiol, and follicle-stimulating hormone (FSH). The expression of E2, Antimullerian hormone (AMH).

Morphological analysis of ovarian tissue

The three groups were sacrificed with ether, bilateral ovaries were taken, dried with filter paper, fixed with neutral formaldehyde, stained with conventional HE, and the number of atresia follicles, growth follicles and the luteal body was observed.

Analysis of protein expression in ovarian tissue

The expression levels of Cysteine-rich protein 61 (CYR61), connective tissue growth factor (CTGF) and caspase-3 protein were assayed in the ovaries of the three groups. The specific operation was to take tissue samples and grind, add cell lysate, wait for 30min to fully lysate, centrifuge the supernatant at 12000r/min at 4°C for 5min, and use BCA protein concentration to detect protein concentration. Then adjust the protein concentration to the same, after boiling for 10min, take 50ug total protein in sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. The classified proteins were transferred to PVDF membrane by a wet membrane transfer device, then 5% skim milk powder solution was given to seal the membrane at 4°C overnight, then TNST buffer solution was washed for 2MI and primary antibodies, namely rabbit monoclonal antibodies against human CYR61, CTGF and Caspase-3, were added respectively. It was sealed at room temperature for 2h, cleaned with TBST solution 3 times, and then added secondary antibody and incubated for 1h without light. Then TBST solution was used 3 times, 10min each time. GAPDH protein was used as the internal reference protein, and the absorbance value of the band was analyzed by gel imaging system. The relative protein expression is the ratio of the absorbance value of the target band to that of the reference protein.

Statistical methods

SPSS20.0 statistical software was used for data processing. Measurement data were expressed as standard deviation ± mean. Comparison between multiple groups was performed by F test. The comparison of counting data was expressed as n or percentage, and the chi-square test was used for inter-group comparison. $P<0.05$ was considered as a statistically significant difference.

Results and discussion

Flow cytometry identification results

The results showed that the mesenchymal stem cells in this study showed high expression of antigen CD105 and CD73, and low expression of CD34 and CD45, proving that they were umbilical cord mesenchymal stem cells (Figure 1).
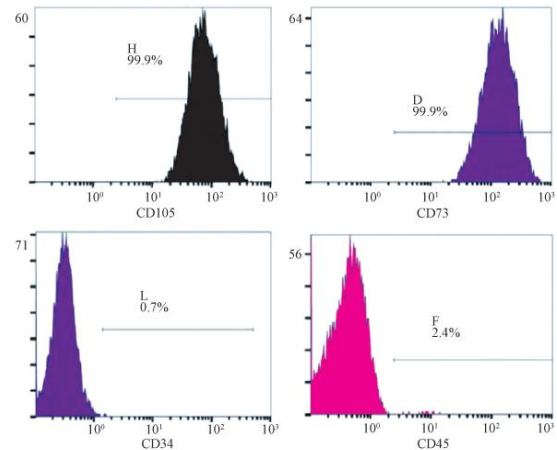

Figure 1. Flow cytometry identification results

Analysis of general conditions of mice

The results showed that the fertility rate in the γ group > α group > β group was statistically significant ($P<0.05$). In terms of litter size and litter weight, α group > γ group > β group, and there were statistically significant differences among groups ($P<0.05$), as shown in Table 1 and Figure 2.

Table 1. Analysis of the fertility of mice

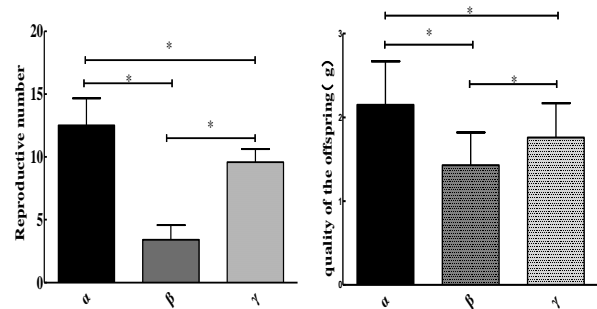| Group	α (n=10)	β (n=10)	γ (n=10)	F	P
Fertility rate	60.00%	40.00%	80.00%	8.542	0.013
Number of births	12.52 ± 2.14	3.41 ± 1.16	9.58 ± 1.05	6.541	0.010
quality of the offspring	2.15 ± 0.52	1.43 ± 0.39	1.76 ± 0.41	7.415	0.009

Figure 2. Evaluation of fertility of mice (*P < 0.05)

Differences in serum sex hormone levels and AMH expression in mice

The results showed that in serum E2 and AMH levels, α group > γ group > β group, and in serum FSH and LH levels, β group > γ group > α group, with statistical significance (P<0.05), as shown in Table 2 and Figure 3.

Table 2. Differences of serum sex hormone levels and AMH expression in mice

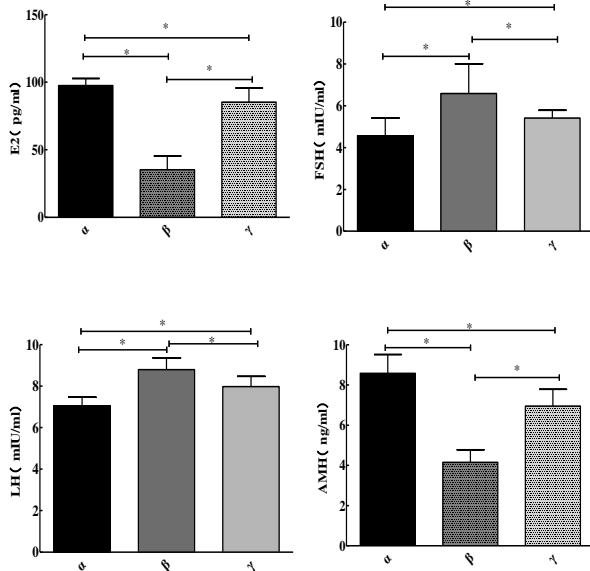| Group	α (n=10)	β (n=10)	γ (n=10)	F	P
E2(pg/ml)	97.48 ± 5.23	35.25 ± 10.16	85.25 ± 10.53	8.416	0.008
FSH (mIU/ml)	4.56 ± 0.85	6.59 ± 1.42	5.41 ± 0.38	10.531	0.001
LH (mIU/ml)	7.05 ± 0.42	8.79 ± 0.57	7.98 ± 0.49	9.775	0.003
AMH (ng/ml)	8.58 ± 0.93	4.15 ± 0.63	6.95 ± 0.83	10.432	0.001

Figure 3. Differences of serum sex hormone levels and AMH expression in mice (*P < 0.05)

Pathological analysis of mouse ovarian sections

HE staining section showed that the ovarian volume of the α group was larger, the follicles at all levels were arranged closely and neatly, and the luteum was well developed, and there was no inflammatory cell infiltration. Compared with the α group, the mice in the β group had smaller, atrophied, and disordered ovaries. Follicles mature many structural malformations, most of them atretic follicles. The number of atresia follicles was decreased and the number of growing follicles was increased in the γ group. The follicular count results showed that the growth follicles in α group > γ group >

β group, and the atretic follicles in β group > γ group > α group, and there was statistical significance among all groups (P<0.05). There was no difference in luteal number among the three groups (P>0.05), as shown in Figure 4 and Table 3.
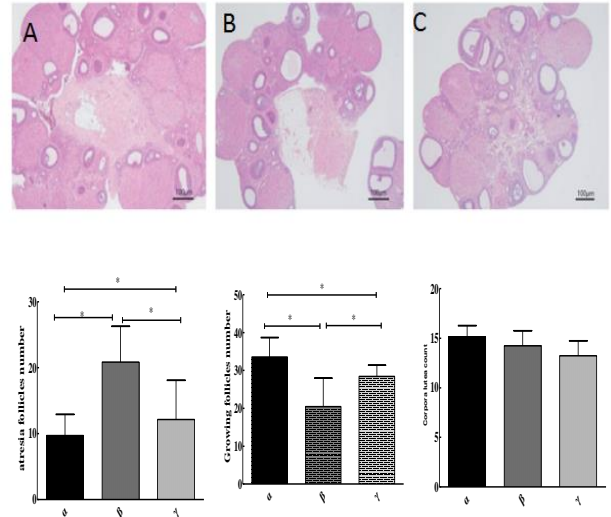

Figure 4. Pathological analysis of mouse ovary sections (A for group α, B for group β, C for group γ, *P < 0.05)

Table 3. Pathological analysis of mouse ovarian sections

Group	α (n=10)	β (n=10)	γ (n=10)	F	P
Number of atresia follicles	9.72 ± 3.21	20.85 ± 5.43	12.16 ± 5.92	8.413	0.008
Number of follicles grown	33.58 ± 5.12	20.45 ± 7.53	28.41 ± 2.99	10.854	0.001
Corpus luteum number	15.14 ± 1.13	14.25 ± 1.52	13.24 ± 1.51	0.842	0.945

Analysis of protein expression in mouse ovarian tissue

The results showed that the expression levels of CYR61 and CTGF in α group > γ group > β group. In caspase-3, β group > γ group > α group had statistical significance (P<0.05), as shown in Table 4 and Figure 5.

Table 4. Analysis of protein expression in mouse ovarian tissue

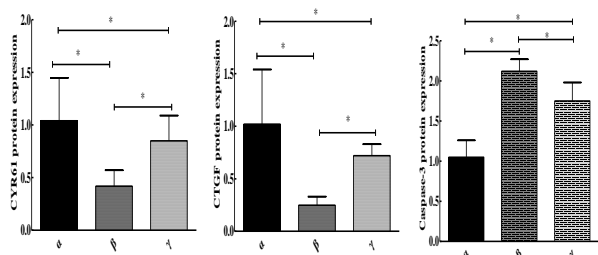| Group	α (n=10)	β (n=10)	γ (n=10)	F	P
CYR61	1.04 ± 0.41	0.42 ± 0.15	0.85 ± 0.24	8.543	0.006
CTGF	1.02 ± 0.52	0.25 ± 0.08	0.72 ± 0.11	10.932	0.001
Caspase-3	1.05 ± 0.21	2.12 ± 0.15	1.75 ± 0.23	8.415	0.008

Figure 5. Protein expression analysis of mouse ovarian tissue (*P < 0.05)

POF is one of the female reproductive health problems and diseases. In recent years, due to the change of lifestyle, living environment and dietary factors, the incidence of POF has gradually increased. The main characteristics of this disease are amenorrhea, reduced estrogen levels, infertility, high gonadotropin levels and lack of mature follicles, while POF patients are also associated with a higher risk of cardiovascular disease, osteoporosis and sexual dysfunction (8). Currently, the etiology and pathogenesis of premature ovarian failure have not been confirmed, and the confirmed etiology includes gene polymorphism, autoimmune, steroidase defect, vaccination, implementation of chemotherapy and radiotherapy, and environmental factors (9). Therefore, due to the complexity of POF disease, there are many therapeutic strategies, but all of them have certain defects, such as hormone replacement therapy, melatonin therapy, immune regulation and stem cell therapy. Among them, stem cell therapy is considered to be the best and most successful cell therapy, mostly because researchers believe that the therapy can simultaneously activate multiple mechanisms, such as nutrition, paracrine, immune regulation and differentiation, and improve all stages of damaged tissues (10, 11). Among stem cell therapies, umbilical cord stem cells were selected for this study because they have fewer ethical issues, are painless to obtain discarded umbilical cords, and lack immunogenicity. Based on the above results, the effects of umbilical cord mesenchymal stem cells on premature ovarian failure and its related mechanisms are discussed.

The results of this study indicated that the fertility rate of the three groups was γ group > α group > β group. In terms of litter size and litter weight, α group > γ group > β group, indicating that the fertility

function of mice with premature ovarian failure was significantly improved under the intervention of mesenchymal stem cells. Yoon et al. (12) showed that the average count of primary and primordial follicles in the EMBRYONIC mesenchymal stem cell group was significantly increased, the count of apoptotic markers of ovarian follicles was significantly reduced, and ovulation was significantly recovered. The blastocyst formation rate from ovulation and the live birth rate per mouse also recovered significantly, suggesting that the embryonic mesenchymal stem cells had restored the structure and function of the cisplatin-damaged ovary. Bahrehbar et al. (13) showed that human embryonic mesenchymal stem cells s significantly restored hormone secretion, survival rate and reproductive function of POF mice, and reduced cell apoptosis of growing follicles. The transplanted mice produced new progeny, suggesting that embryonic mesenchymal stem cells can restore the structure of damaged ovarian tissue and its function in chemotherapy-induced damaged POF mice and save fertility. The above research results are consistent with the results of this study, which all confirm that mesenchymal stem cell therapy can improve the reproductive function of mice with premature ovarian failure. The specific reason may be that mesenchymal stem cells can improve ovarian function through paracrine form according to the current research results. That is, the vesicle contents secreted by the cell contain cytokines, growth factors, signaling lipids, mRNA and regulatory mRNA. The factors mentioned above are involved in changes in intercellular communication, signal transduction and tissue metabolism. If studies have shown that MESenchymal stem cells can regulate the expression of GCSF to reduce apoptosis of granulosa cells (14). Other studies have shown that mesenchymal stem cells can differentiate into oocyte structure and express germ cell-specific mRNA and protein markers, increase the growth of damaged endometrial mechanism cells, and secrete vascular growth factor and anti-apoptotic factors to repair endometrial damage (15). In conclusion, according to the current animal experiments, mesenchymal stem cells are effective in the treatment of premature ovarian failure.

Secondly, in terms of changes of sex hormone and AMH levels, the results of this study showed that in serum E2 and AMH levels, α group > γ group > β

group, and in serum FSH and LH levels, β group > γ group > α group, suggesting that mesenchymal stem cell intervention can effectively improve the secretion of sex hormone in mice with premature ovarian failure. Shen et al. (16) showed that umbilical cord mesenchymal stem cells could repair ovarian tissue damaged by chemotherapy to a certain extent, improve the degree of apoptosis of ovarian tissue and improve the endocrine function of mice ovary. Liu et al. (17) showed that amniotic mesenchymal stem cell transplantation can improve the damaged ovarian tissue structure and function of oxidized POF mice, and the specific mechanism is related to the promotion of follicular development, granulosa cell proliferation and secretion function by improving the local ovarian microenvironment. The results with the results of this study is consistency, both confirmed that umbilical cord mesenchymal stem cells between ovarian premature aging intervention can improve the secretion of sex hormones and AMH, specific reasons may be because the follicle growth and survival of the endocrine system to produce a variety of steroid hormone regulation, and hormone secretion disorder is not the only source of PDF disease pathological process, but the inevitable result. FSH is produced by adenohypophysis, and FSHR is mainly expressed in granulosa cells. Therefore, the increase of FSH levels can stimulate the growth of follicles. The growing follicle can produce E2, which produces negative feedback to FSH, thus avoiding excessive follicle growth (18). The application of cyclophosphamide, the chemotherapeutic drug used in this study, usually leads to the apoptosis of granulosa cells and the decrease of E2 levels. The decrease in E2 negative feedback leads to an increase in FSH levels, which in turn leads to premature ovarian failure. However, AMH is expressed by granulosa cells in primary and early sinus follicles and usually acts as an inhibitory factor in the ovary, and its level is usually closely related to the size of the follicular cistern (19). In this study, mesenchymal stem cells can secrete a variety of cytokines through paracrine, such as VEGF, HGF and other cytokines, to repair ovarian tissue damage, and reduce the apoptosis of ovarian mesenchymal cells and granulosa cells through the mechanism of anti-oxidative stress, thus improving the quality of oocytes. Or differentiated into similar solid cells to maintain the integrity of follicular development

microenvironment, and the above mechanisms can improve the apoptosis of granulosa cells, continue the negative feedback of E2, and then reduce FSH. The integrity and enlargement of follicular cisterns further increased AMH levels.

Finally, in the pathological analysis of ovarian tissue and protein expression, the results showed that the growth of follicles in α group > γ group > β group. β > γ > α group was observed in atresia follicles, α > γ > β group was observed in CYR61 and CTGF protein expression, β > γ > α group was observed in caspase-3. These results indicate that the ovarian function of mice can be significantly recovered with the intervention of mesenchymal stem cells, and the proliferation and apoptosis of human granulosa cells and follicular cells can be promoted and reduced by up-regulating CYR61 and CTGF and down-regulating capase-3, thus changing follicular development. According to the current research results, few studies are exploring mesenchymal stem cells and CYR61 and CTGF, but most studies have confirmed the effect of mesenchymal stem cells on caspase-3 apoptosis proteins. Zhang et al. (20) showed that placental mesenchymal stem cell transplantation can significantly improve serum gonadotropin and low estrogen levels in POF mice, promote follicular development, inhibit excessive follicular atresia and granulosa cell apoptosis, and improve ovarian reserve capacity, which may be achieved by increasing the expression of AMH and FSHR in the ovary. Wang et al. (21) showed that placental mesenchymal stem cell transplantation also reduced apoptosis of granulosa cells and promoted proliferation of granulosa cells, but all these improvements were dose-dependent, and the transplanted mesenchymal stem cells up-regulated the expression of Bcl-2, AMH and FSHR in the ovary of POF rats, and down-regulated the expression of caspase-3. The results of this study were consistent with those of the above studies, which confirmed that the application of MESenchymal stem cells could down-regulate caspase-3. The specific reason was related to the improvement of the ovarian microenvironment by mesenchymal stem cells intervention and the reduction of apoptosis of granulosa cells and stromal cells. Both CYR61 and CTGF are extracellular matrix-related proteins, and current studies related to the ovary have confirmed that the high expression of CYR61 is related to the

development and poor prognosis of ovarian cancer (22). Some studies have also shown that CYR61 is regulated by estrogen in the endometrium (23). Some studies have also confirmed that CYR61 and CTGF can jointly promote the growth of VEGF to promote the generation of blood vessels (24). Some studies have also confirmed that E2 can up-regulate the expression of CTGF (25-28). In conclusion, this study speculated that the increase of CYR61 and CTGF may be related to the vesicles secreted by mesenchymal stem cells. For example, Wang et al. (29) confirmed that Mir-24 carried by HMC-EVS plays a protective role in I/R injury. Perhaps by targeting AQP4 and activating the P38 MAPK/ERK1/2/P13K/AKT pathway. CYR61 is also a downstream factor of P13K signaling pathway, so it is speculated that mesenchymal stem cells may enhance angiogenesis and restore ovarian function by directly increasing CYR61 or by P13K/CYR61 signaling pathway. Therefore, the specific molecular mechanism needs to be further explored.

In conclusion, intervention with umbilical cord mesenchymal stem cells can significantly improve the expression levels of CYR61 and AMH, reduce the level of FSH, promote cell survival, improve the reproductive quality of mice, and restore the physiological function of the ovary. Umbilical cord mesenchymal stem cells have high feasibility in the treatment of premature ovarian failure.

Acknowledgement

Conflict of interest

The authors declare no conflict of interest.

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