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Research Article

Effect of Human Umbilical Cord Mesenchymal Stem Cells on Ovarian Function in Lupus Mice

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Abstract

Background: Systemic Lupus Erythematosus (SLE) is an autoimmune disease that occurs in women of childbearing age. Clinical treatment of SLE is associated with a high incidence of premature ovarian failure, which is attributed to cyclophosphamide gonadal toxicity; however, the role of immune abnormalities, if any, remains unclear.

Material and Methods: In this study, we used MRL/lpr mice to observe changes in ovarian structure and secretory function in SLE. Further, lupus and control mice were injected with human umbilical cord mesenchymal stem cells (HUC-MSCs) *via* the tail vein at weeks 12 and 16; serum was collected at week 20, and serum estradiol (E2), Follicle Stimulating Hormone (FSH), and ELISA determined Anti-Mullerian Hormone (AMH) levels. HE staining was used to observe the morphology of the ovary and count all levels of follicles. Hepatocyte Growth Factor (HGF) and Independent Games Festiva-1(IGF-1) expression in the ovarian tissue was observed by immunohistochemistry.

Results: Ovarian function in lupus mice was abnormal, as indicated by decreased serum E2 and AMH levels, and increased FSH level. HUC-MSCs transplantation could cause a significant up-regulation of serum E2 and AMH, down-regulation of FSH, improved follicular development, inhibition of follicular atresia, and enhanced ovarian reserve capacity. Immunohistochemically results showed that expression of IGF-1 and HGF increased after HUC-MSC transplantation.

Conclusion: HUC-MSC transplantation could promote IGF-1 and HGF expression in the ovarian tissue of SLE mice, which could be a mechanism by which HUC-MSCs improve ovarian function of lupus mice.

Keywords: Human umbilical cord mesenchymal stem cells; Systemic lupus erythematosus; Premature ovarian failure; MRL/lpr mice

Introduction

Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease that occurs in women of childbearing age. The incidence of Premature Ovarian Failure (POF), which seriously affects reproductive function and consequently mental health, is significantly higher in women with SLE than in healthy women. Therefore, protection of ovarian function in patients with SLE is of great clinical interest [1]. Gonadal toxicity of Cyclophosphamide (CTX) treatment is a common cause of POF in patients with SLE [2]; however, immune abnormalities could also be involved [3]. The involvement and role of abnormal immune function in POF remains unclear. Mesenchymal Stem Cells (MSCs) have shown significant safety and efficacy in the treatment of POF in animal models [4]. However, the use of MSCs to treat POF in lupus mice has not been reported. Human umbilical cord mesenchymal stem cells (HUC-MSCs) are capable of several functions including self-renewal, multi-directional differentiation, and immune regulation. They are also easy to obtain and are not associated with ethical concerns. HUC-MSCs have been used in the clinical treatment of several diseases, including refractory lupus nephritis [5]. The main aim of this study was to examine ovarian function, and the occurrence of POF in lupus mice, and subsequently determine the effects of HUC-MSCs transplantation on ovarian function in lupus mice. We also aimed to identify potential mechanisms underlying these effects, to provide a theoretical basis for the development of novel HUC-MSCs-based strategies for the treatment of POF in patients with SLE.

Materials and Methods

Experimental animals and HUC-MSC transplantation protocol

We used 30 SPF female MRL/lpr mice (6 weeks old; Shanghai Lingchang Biotechnology Co., Ltd., license number: SCXK [Shanghai] 2013-0018) and 6 SPF female C57BL/6 mice (6 weeks old; Experimental Animal Center of Jiangsu University, license number: SCXK (Jiangsu) 2013-0011). All experimental mice were raised in the SPF-class breeding area of the Animal Experiment Center of Jiangsu University until they were 20 weeks old. The Animal Experiment Ethics Committee of Jiangsu University approved all animal experiments. The MRL/lpr mice were randomly divided into the MSC1 (n = 6), MSC2 (n = 6), PBS1 (n = 6), PBS2 (n = 6), and model control (n = 6) groups; C57BL/6 mice (n = 6) were used as normal controls. The MSC1 group was injected with passage 3 (P3) HUC-MSCs (1×10^7 cells/mL, 0.1 mL/10g body weight) *via* the tail

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vein at 12 weeks, and the MSC2 group was injected with P3 HUC-MSCs (1×10^7 cells/mL, 0.1 mL/10g body weight) *via* the tail vein at 12 weeks and 16 weeks, the PBS1 group, the control for the MSC1 group, was injected with the same volume of PBS at 12 weeks, and the PBS2 group was injected with the same volume of PBS at 12 and 16 weeks as the control for the MSC2 group. All animals were sacrificed at 20 weeks, and serum and ovaries were collected. The ovaries were fixed in 10% formaldehyde.

Detection of serum hormone levels by elisa

The concentration of serum FSH, E2, and AMH was determined using an ELISA kit (Nanjing Jiancheng Institute of Bioengineering Co., Ltd.) according to the manufacturers' instructions.

Ovarian histomorphology and follicular count

After the mice were sacrificed, the ovarian tissues were separated, fixed in formaldehyde fixative, embedded in paraffin, and stained with HE. The ovarian structure was observed under a microscope, and the number of follicles at various stages noted. Follicular grading criteria were: primordial follicles: oocytes enclosed by a single layer of flat granular cells; primary follicles: oocytes enclosed by one or more layers of cubic granular cells with zona pellucida between the two; secondary follicles: appearance of follicular cavities, some forming cumulus, and two layers of follicular membrane; atresia follicle: collapsed follicular wall, absent or unclear structure of the egg cell, and shrunken zona pellucida.

Immunohistochemical staining of ovarian tissue

After the mice were sacrificed, the ovarian tissues were separated. Fresh ovarian tissues were embedded in OCT and cut into 5-μm-thick sections for HGF and IGF-1 immunohistochemically staining. Immunohistochemistry was performed by SP method, according to manufacturers' instructions.

Statistical analysis

SPSS 20.0 statistical software was used for analysis. The data in accordance with normal distribution were expressed as mean ± standard deviation (x ± s). *T*-test was used for comparison between the two groups, one-way ANOVA was used for comparison among groups, and Dunnett's T3 test was used for paired comparison. $P < 0.05$ was considered statistically significant.

Results

Changes in ovarian function in lupus mice

We evaluated ovarian function in the mice by measuring the serum levels of E2, FSH, and AMH in each experimental group. Serum E2 levels (296.75 ± 61.42 *vs.* 117.73 ± 25.87, $P < 0.01$) and AMH levels (308.41 ± 48.64 *vs.* 116.75 ± 31.10, $P < 0.001$) were significantly lower, and FSH levels (191.21 ± 76.80 *vs.* 742.80 ± 100.63, $P < 0.001$) were significantly higher in the MRL/lpr lupus group than in the C57BL/6 group.

We used HE staining to observe ovarian structural changes, and found that the follicles of the normal group of mice grew actively and were clearly visible. The follicles in the model group were significantly fewer, disorderly in arrangement, and with different shapes. The number of follicles in the treatment group was higher than that in the model group, and the level was clear, which was second to the shape of the control group. The number of primordial follicles (7.00 ± 1.83 *vs.* 2.50 ± 1.29, $P < 0.01$), primary follicles (10.25 ± 1.89 *vs.* 5.50 ± 1.29, $P < 0.01$), and secondary follicles (6.50 ± 2.08 *vs.* 2.00 ± 0.82, $P < 0.01$) were significantly lower, and the number of atresia follicles (1.00 ± 0.82 *vs.* 2.75 ± 0.96, $P < 0.05$) significantly higher, in the MRL/lpr lupus mice than in the C57BL/6 mice.

Effect of HUC-Mscs transplantation on the ovarian function of lupus mice

The differences in serum E2, FSH, and AMH levels between the PBS1, MSC1, PBS2, and MSC2 groups were statistically significant (Table 1). Serum E2 ($P < 0.001$) and AMH ($P < 0.05$) levels were significantly higher, and serum FSH level ($P < 0.001$) significantly lower, in the MSC1 group than in the PBS1 group. Serum E2 ($P < 0.001$) and AMH ($P < 0.01$) levels were significantly higher, and serum FSH level ($P < 0.001$) significantly lower in the MSC2 group than in PBS2 group. There was no significant difference in serum E2 and FSH levels between the MSC1 and MSC2 groups ($P > 0.05$). The serum AMH level in the MSC2 group was significantly higher than that in the MSC1 group ($P < 0.05$).

Effect of HUC-Mscs transplantation on ovarian morphology of lupus mice

The differences in follicle counts at all stages between the PBS1, MSC1, PBS2, and MSC2 groups were statistically significant (Table 2). The number of primordial, primary, and secondary follicles was significantly higher ($P < 0.05$), and the number of atretic follicles significantly lower ($P < 0.05$), in the MSC1 group than in the PBS1 group. The number of primordial, primary, and secondary follicles ($P < 0.05$) was significantly higher, and the number of atretic follicles significantly lower ($P < 0.05$), in the MSC2 group than in the PBS2 group. There was no significant difference in the number of primordial, primary, and secondary follicles between the MSC1 and MSC2 groups ($P > 0.05$). The number of primordial follicles in the MSC2 group was significantly higher than that in the MSC1 group ($P < 0.05$). Thus, multiple infusions of HUC-MSCs could significantly increase the number of primordial follicles and could therefore be more efficacious in improving ovarian function.

Effect of HUC-Mscs transplantation on HGF and IGF-1 expression in the ovarian tissue of lupus mice

In order to elucidate the mechanisms by which HUC-MSCs

Table 1: Changes in sex hormone levels in the experimental groups.

	PBS1	PBS2	MSC1	MSC2	F	P
E2 (ng/dl)	153.38±13.48	153.78±31.97	622.74±129.32	769.33±100.99	43.38	<0.001***
FSH (mIU/dl)	626.00±76.60	589.74±63.31	310.15±99.62	190.86±32.50	32.77	<0.001***
AMH (ng/dl)	122.05±71.70	177.12±32.47	259.04±83.10	433.73±77.22	11.65	0.003**

Effect of HUC-MSC transplantation on ovarian function in lupus mice. ELISA was used to detect the levels of sex hormones in each experimental group. The MSC1 group was injected with P3 HUC-MSCs and the PBS1 group was injected with the same volume of PBS once at 12 weeks. The MSC2 group was injected twice with P3 generation HUC-MSCs and the PBS2 group was injected with the same volume of PBS at 12 and 16 weeks. Data were expressed as means ± SD, (n=6).

Table 2: Ovarian follicle counts in the experimental groups.

	PBS1	PBS2	MSC1	MSC2	F	P
Primordial follicle	2.75±0.96	3.75±1.26	6.50±1.29	9.50±3.11	10.627	0.001**
Primary follicle	5.25±3.10	4.75±3.30	12.50±3.11	11.75±2.06	7.93	0.004**
Secondary follicle	2.00±0.82	2.25±1.26	5.00±1.41	4.50±1.29	6.352	0.008**
Atretic follicle	4.00±0.82	4.75±2.99	1.25±0.96	1.00±0.82	5.194	0.016*

Comparison of ovarian follicle count in each experimental group. Counts of ovarian follicle follicles were presented as means± SD (Primordial follicle, primary follicle, secondary follicle, atretic follicle of POF each experimental group versus HUC-MSCs transplanted group, n=6, P<0.05).

could improve ovarian function, we used immunohistochemistry to detect the expression of HGF and IGF-1 in the ovarian tissue of lupus mice. HGF and IGF-1 expression increased after HUC-MSC transplantation; however, the difference was not statistically significant. Thus, HUC-MSCs might promote the expression of HGF and IGF-1 in the ovarian tissue. However, the specific mechanism remains unclear.

Discussion

Systemic Lupus Erythematosus (SLE) is an autoimmune disease that occurs in women of childbearing age, and most patients with SLE present with abnormal reproductive function [6]. However, the specific cause of these abnormalities is unclear. Autoimmune oophoritis caused by the disease itself could lead to premature ovarian failure [7], and the use of drugs with gonadal toxicity such as cyclophosphamide can cause premature ovarian failure, and therefore premature amenorrhea and infertility [8]. The chronic inflammatory state of the disease interferes with the function of the Hypothalamic-Pituitary-Gonadal Axis (HPA) [9], causing abnormal ovarian function and the associated antiphospholipid antibody syndrome causes miscarriage, premature delivery, and stillbirth [10]. However, the exact mechanism underlying POF in patients with SLE remains unclear, and there are no reports of *in vivo* studies on the topic. In this study, the ovarian function of lupus mice was studied to determine whether SLE itself may cause abnormal ovarian function. Our results confirm a role for immune dysfunction in POF in patients with SLE. However, the sample size was limited, and further studies with larger experimental groups are warranted.

Heterogeneity of POF is also reflected by the variety of possible causes, including autoimmunity, drugs, toxics, radiation, and infectious as well as genetic defects [11]. Our animal experiments proved that SLE itself likely causes ovarian dysfunction; however, whether SLE causes POF remains unclear. A cross-sectional study to assess the incidence of POF in 961 female patients with SLE showed that the incidence of POF in patients with SLE who did not receive cyclophosphamide treatment was not significantly lower than that in healthy individuals. This suggested that the autoimmune response in patients with SLE might not involve the ovary [12]. However, some clinicians have observed that the ovarian reserve function of premenopausal women with SLE for the first time is lower than that of healthy women [13]. Compared with healthy women, patients with SLE of childbearing age who have never received any drug treatment have lower serum AMH level, higher incidence of abnormal menstruation, imbalance of lymphocyte ratio, a lower proportion of CD4+ T lymphocytes and NK cells, and a higher proportion of B cells and CD8+ T lymphocyte, which suggested that abnormal autoimmune function in SLE might lead to the impairment of ovarian reserve function [14]. In order to elucidate the status of ovarian function in SLE, we studied the ovarian function of lupus mice. We assessed the ovarian reserve function by measuring serum E2, FSH, and AMH levels, and follicle counts at all stages. E2 is a sex hormone synthesized and secreted by the ovaries. A decrease in E2 levels indicates a decline in ovarian secretion of estradiol. FSH is a gonadotropin secreted by adenohypophysial follicle stimulating hormone cells, and can promote the synthesis and secretion of estrogen, maturation of follicles, and ovulation. Increases in FSH levels indicate a decline of ovulation function. AMH is secreted by ovarian granulosa cells and promotes the development of gonads. AMH concentration is not affected by gonadotropins and does not change with the menstrual cycle. It is therefore considered the most reliable indicator for evaluating ovarian reserve. In our study, E2 and AMH levels and follicle count were significantly lower in lupus mice than in normal mice, FSH level and number of atresia follicles were significantly higher in lupus mice than in normal mice, which was consistent with clinical reports of ovarian reserve dysfunction in patients with SLE [13-15]. Therefore, the disease itself could cause POF, which could be exacerbated with the use of drugs with gonadal toxicity, such as cyclophosphamide. Ovarian function of patients with SLE should therefore be protected during clinical treatment.

Several methods, including Hormone Replacement Therapy (HRT), Gonadotropin-Releasing Hormone analog (GnRH-a), melatonin supplementation, dehydroepiandrosterone supplementation, and cryopreservation of eggs, embryos, or ovarian tissue, are currently used to treat POF. However, these treatments have poor clinical efficacy and several limitations. Combined hormone replacement therapy is most common; however, an optimal treatment plan and method of administration has not been established, and this strategy is associated with risks of vaginal atrophy, venous thrombosis, and tumors [16]. The use of GnRH-a during cyclophosphamide treatment could significantly reduce the incidence of POF in young women with SLE [17], and GnRH-a could therefore be used to prevent premature ovarian failure in premenopausal women with SLE receiving cyclophosphamide treatment [18]. EULAR recommendations on family planning, assisted reproduction, pregnancy, and menopause management in female patients with SLE state that GnRH-a should be considered to preserve fertility before using alkylating agents [19]. Cryopreservation of eggs, embryos, or ovarian tissues is expensive, technically demanding, and rely on assisted reproductive technology. These fertility preservation methods are mainly suitable for young female patients with reproductive requirements and have limited clinical applications [20,21]. Therefore, there is an urgent need for safe and effective methods to treat premature ovarian failure in patients with SLE.

Stem cells are capable of self-replication and multidirectional

differentiation. They can regenerate and repair body tissue, and regulate the body's immune function *via* mechanisms such as cytokine secretion. Stem cell transplantation is therefore considered a therapeutic method to repair damaged ovarian function [22]. Mesenchymal Stem Cells (MSCs) are pluripotent stem cells. Compared with embryonic stem cells and hematopoietic stem cells, MSCs have low immunogenicity and strong immunosuppressive activity. MSCs can restore ovarian function through a variety of mechanisms, including promoting the expression of various cytokines through paracrine pathways, improving the ovarian microenvironment, inhibiting granulosa cell apoptosis, and self-differentiation into estrogen producing cells [23-26]. The HUC MSCs used in this study were derived from the umbilical cord tissue of healthy newborns. The umbilical cord is considered medical waste, and therefore has the advantages of a non-invasive sampling process, availability, low cost, and no associated ethical issues. HUC-MSCs show strong immune regulation and damage repair functions, stable biological properties, significant effects in the treatment of autoimmune diseases [27-28], and great clinical application potential in the treatment of premature ovarian failure [29]. In this study, HUC-MSC transplantation treatment could significantly improve the ovarian reserve in lupus mice, increase serum E2 and AMH levels, reduce serum FSH levels, increase the number of primordial follicles, primary follicles, and secondary follicles, and reduce the number of atretic follicles. Our results therefore provide a theoretical basis for the development of novel strategies for the clinical treatment of patients with SLE and ovarian failure, particularly women of childbearing age who have fertility requirements. For patients with SLE who choose cyclophosphamide therapy, preventive treatment could be considered; however, the preventive effects require extensive further study.

The role of stem cells in the treatment of premature ovarian failure is unclear. Human Placental Mesenchymal Stem Cells (hPMSCs) could promote ovarian function by secreting Epidermal Growth Factor (EGF) [30]. HUC-MSCs could improve ovarian endocrine function by modulating ovarian cell apoptosis [31]. Moreover, HUC-MSCs could ameliorate premature ovarian failure by regulating the autophagy of ovarian cells and the expression of CD8+CD28- T cells in peripheral blood [32], and HUC-MSCs-derived exosomes miR-17-5P could promote the functional recovery of POF induced by cyclophosphamide by regulating the expression of related genes [33]. Thus, HUC-MSCs can repair ovarian function in a variety of ways; however, the main mechanism is not completely clear. HUC-MSCs can survive in mouse ovarian tissue for 8 weeks [34], and can secrete HGF and IGF-1. HGF is an important factor in the environment of follicles, and can accelerate the viability of growing follicles and promote the proliferation of ovarian surface epithelium to supplement the damaged areas formed during ovulation. IGF-1 is expressed in granulosa cells and healthy follicles, but cannot be detected in atretic follicles, and is an important regulator of granulosa cell proliferation in the early stages of follicular development. The expression of ovarian IGF-1 stimulates the production of progesterone and estradiol, and enhances the responsiveness of FSH in granular cells by improving the expression of FSH receptors. Therefore, IGF-1 is an important indicator of ovarian function. We studied the changes in ovarian protein expression before and after HUC-MSCs transplantation to identify the mechanisms by which HUC-MSCs could improve ovarian function. IGF-1 and HGF expression in the premature ovarian failure lupus mouse groups that received HUC-MSCs transplantation slightly increased. Serology suggested that upregulation of IGF-1 and HGF in ovarian tissue could be a mechanism by which HUC-MSCs improve ovarian function. However, how HUC-MSCs act on ovarian tissue to promote IGF-1 and HGF expression requires further study.

Conclusion

In this study, we found that SLE may cause abnormal ovarian function, mainly manifested as changes in ovarian structure and secretory function. These findings require validation in clinical trials. HUC-MSC transplantation could improve ovarian function, repair ovarian tissue structures, and enhance ovarian secretion function. Our results suggest novel strategies to treat patients with SLE with premature ovarian failure. The repair function of HUC-MSCs could include a paracrine mechanism involving HGF and IGF-1. However, the specific mechanism remains unclear and merits further study.

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