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Theriogenology

Intravenous human endothelial progenitor cell administration into aged mice enhances embryo development and oocyte quality by reducing inflammation, endoplasmic reticulum stress and apoptosis

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ABSTRACT. Stem cell therapy has been proposed to restore the function and structure of injured tissues. In the present study, we investigated the ability of human endothelial progenitor cells (hEPCs) to attenuate ovarian aging and dysfunction. Female ICR mice aged 4 and 6 months were injected with cultured hEPCs. Cultured hEPCs were injected intravenously twice with 5×10^4 cells with a 4 day interval. After pregnant mare serum gonadotropin and human chorionic gonadotropin stimulation, oocytes and ovaries of aged mice were collected, cumulus-free oocytes were activated by $SrCl_2$ and gene expression levels related to inflammation, apoptosis, follicle development and endoplasmic reticulum (ER) stress in ovaries were compared. Administration of hEPCs attenuated the level of inflammatory cytokines and adverse apoptotic factor, as well as reducing ER stress in the ovaries. Increased cleavage and blastocyst formation rates and cell numbers in blastocysts from hEPCs-treated aged mice vs. same aged control mice demonstrated a protective function of hEPCs against reproductive aging. Based on these data, we suggest that treatment with hEPCs attenuates reproductive aging and dysfunction potentially via regulation of inflammation, apoptosis and ER stress.

KEY WORDS: endoplasmic reticulum stress, human endothelial progenitor cells, inflammation, ovarian aging

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An age-related mouse model exhibits infertility symptoms resembling those found in older women and has been widely used in previous studies [26, 27]. Previous reports suggested that age-associated oxidative stress results in follicular atresia and a decline in the number and quality of oocytes [33], which is an important contributor to the progression of reproductive aging. Furthermore, oxidative stress and endoplasmic reticulum (ER) stress are linked to multiple pathologies including apoptosis and inflammation.

Stem cell administration constitutes a promising new strategy in the treatment of infertility or chemotherapy induced ovarian damage. Several studies have described the beneficial effect of treatment with bone marrow or mesenchymal stem cells (MSCs) transplantation in animal models of chemotherapy-induced ovarian damage and infertility [13, 23, 32]. These results suggested the possibility and the potential of stem cells to treat ovarian failure. In clinical studies, successful pregnancies in women after stem cell transplantations are well documented [3, 16, 19]. Similarly, it was reported that 85% of pregnancies after stem cell transplantation resulted in live births [30] which demonstrates the feasibility and safety of stem cell transplantation. Furthermore, a recent study with human umbilical cord mesenchymal stem cells revealed that they up-regulated anti-apoptotic signals as well as anti-ER stress signals [40]. Several anti-oxidative agents used to treat infertility have been examined in detail and their application may represent a successful strategy for therapy or improvement of infertility including early or unknown reasons for infertility [26, 27]. Therefore, anti-ER stress or apoptotic therapy may be a crucial approach for delaying age-associated infertility.

Human endothelial progenitor cells (hEPCs) promote vascular repair by migration, homing into target tissues and incorporating

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neovascularization, thereby offering great promise for a novel treatment approach. Various diseases such as diabetes [10] as well as polycystic ovary syndrome [20] are related to EPCs dysfunction, introducing additional challenges for autologous EPCs therapy. Some complications of pregnancy including pre-eclampsia, hypertension and gestational diabetes are also associated with systemic endothelial dysfunction [9, 17]. To address this issue, we reasoned that hEPCs may serve as an alternative therapy for reproductive aging because they are easily harvested from human blood and have the ability to repair damaged blood vessel walls and to form new vasculature. The potential advantages of hEPCs for treating or delaying ovarian aging are further indicated by their use in regenerative medical areas to repair damaged tissues resulting from traumas such as stroke [11], kidney injury [6], myocarditis [40] and bleomycin-induced bronchopulmonary dysplasia [1]. In this study, we evaluated the efficacy of hEPCs in an aged mouse model and investigated the mechanism of this potential therapeutic or improvement effect. We investigated whether an age-dependent fertility decline was exhibited in ICR mice. We also investigated whether intravenous hEPCs can effectively prevent reproductive aging and ovarian aging-associated infertility in mice, as well as evaluating the potential underlying mechanisms.

MATERIALS AND METHODS

Animals and ethics statement

ICR mice aged 2, 4 and 6 months old were purchased from the Joong-Ang Animal Research Center. Mice were housed under a 14–10 hr light-dark cycle at 25 ± 2°C with food and water provided *ad libitum*. The Institutional Animal Care and Use Committee of Seoul National University approved the experimental protocol and recommendations described in "The Guide for the Care and Use of Laboratory Animals" published by the Institutional Animal Care and Use Committee of Seoul National University (approval number; SNU-150331-4). In this respect, the facilities and procedures performed met or exceeded the standards established by the Committee for Accreditation of Laboratory Animal Care at Seoul National University.

Isolation and culture of human endothelial progenitor cells

Isolation of hEPCs was performed according to the previously reported protocol. In brief, peripheral blood was acquired from 4 healthy human donors [4] with permission from the donors and the Institutional Review Board at Biostar (RBIO 2015-12-001). Using Ficoll-Hypaque (GE Healthcare Life Science, Piscataway, NJ, U.S.A.), and after density gradient centrifugation at 2,500 g for 30 min, peripheral blood mononuclear cells (PBMCs) were separated and [14, 29] were obtained. Separated PBMCs at a concentration of 1–3 $\times 10^7$ cells were seeded into a fibronectin-coated T25 flask with Defined Keratinocyte-SFM (Gibco, Grand Island, NY, U.S.A.)-based culture medium containing 0.2 mM ascorbic acid, 10 μg/m*l* L-glutamine, 10 *n*g/m*l* human epidermal growth factor, 5 μg/m*l* insulin, 1 *n*g/m*l* selenium, 74 *n*g/m*l* hydrocortisone, 5 *n*g/m*l* Lin28, 1% antibiotic-antimycotic and 10% fetal bovine serum. The cells were incubated at 37°C under 5% CO_2 and the medium was changed on day 2 after seeding and then replaced twice a week. EPC colony formation appeared after 2–4 weeks of incubation. EPC colonies were passaged to T25 flasks or 6-well plates depending on each colony size. Isolated EPCs were passaged when they reached 80% to 90% confluence. Every two days, non-adherent cells were removed and 2 m*l* of fresh medium was added.

Cell transplantation

In brief, a suspension of 5×10^4 hEPCs in 30 μl of sterilized PBS was injected into the tail vein of each mouse in age groups 4 and 6 months old. Mice in the same age groups with PBS injection only were designated as controls. The injection with hEPCs from the same donor as the 1st injection or another donor was repeated 4 days later. Passages 3–5 of hEPCs were used for all the transplantation experiments.

Collection of oocytes and parthenogenetic activation

In initial experiments, female mice 2, 4 and 6 months old (10 per age group) were maintained. Females were injected with 7.5 IU pregnant mare serum gonadotropin (PMSG, Calbiochem, La Jolla, CA, U.S.A.) and then 7.5 IU human chorionic gonadotropin (hCG, Sigma) 48 hr later. Mice were euthanized by cervical dislocation.

For investigating the effect of hEPCs, on the day following the second EPCs injection, mice were super-ovulated by intraperitoneal injection of 5 IU PMSG, followed by 7.5 IU hCG 48 hr later. The cumulus-oocyte complexes (COCs) were isolated from the ampullary portion of the oviduct 14 hr after the hCG injection. Cumulus cells of COCs were removed by incubation for 1 min with potassium simplex optimized medium (KSOM) containing 0.1% hyaluronidase. Oocytes were stimulated in KSOM with $SrCl_2$, EGTA and cytochalasin B. Putative embryos were cultured in 20 μl droplets of KSOM in a humidified atmosphere of 5% CO_2. Cleavage and embryo development were examined every 20–24 hr, and the numbers of cleaved embryos at 20–24 hr and blastocysts at 120 hr were counted. To count total cell numbers in blastocysts, embryos were stained with Hoechst 33324 and observed under fluorescence microscopy (Olympus, Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR) assay

Total RNA was extracted from ovarian tissue using the easy-spinTM (DNA-free) Total RNA extraction Kit (iNtRON Biotechnology, Inc., KyungGi-Do, Korea) and RNA was used for cDNA synthesis using Maxime RT-PCR Premix (iNtRON Biotechnology, Inc.). A NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.) was used to quantitatively measure the total RNA and synthesized cDNA concentration. PCR reactions were set up in duplicates using the Universal SYBR Green Master (TaKaRa, Kusatsu, Japan), and run on the StepOne^{TM} Real-Time PCR System (Applied

Table 1. Effect of age on ovulation and embryo development in ICR mice stimulated with PMSG and hCG at 2, 4 and 6 months old

	No. mice	Total no. ovulated oocytes (Mean ± SEM, per mice)	*In vitro* development		Total blastocyst cell no.
			No. cleaved embryos	No. blastocyst	
2 months old	12	359 (29.9 ± 3.1)	318 (87.1 ± 2.8)[a)]	171 (47.0 ± 5.2)[a)]	94.9 ± 4.8[a)]
4 months old	9	171 (19.0 ± 3.2)	122 (66.6 ± 4.8)[b)]	36 (17.1 ± 5.4)[b)]	61.6 ± 6.4[ab)]
6 months old	9	170 (18.9 ± 4.1)	69 (40.7 ± 8.5)[c)]	23 (14.3 ± 3.5)[b)]	44.5 ± 4.8[b)]

Results are presented as the mean ± SEM of 9–12 mice per group. Different superscripts within same column represent significant differences (P<0.05) among groups.

Biosystems, Waltham, MA, U.S.A.). Each sample was repeated three times and analyzed with 18s as the internal control. The final PCR reaction volume of 20 μl contained 10 μl SYBR Green PCR Master Mix (Applied Biosystems), 1 μl cDNA template, 0.4 μl (10 pmol/μl) forward primer, 0.4 μl (10 pmol/μl) reverse primer and 8.2 μl water. Amplification was conducted with 10 min consisting of an initial denaturation step at 95°C, followed by 40 cycles of denaturation for 15 sec at 95°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C. All steps of the oligonucleotide primer sequences are described in previous reports [22, 26]. Amplification data from three replicates were collected and analyzed using the $2^{-\Delta\Delta Ct}$ method. For ease of comparison, the average expression level of each gene from the control group was set as one.

Western blotting analysis

Ovaries were lysed using a pro-PREP protein extraction solution (iNtRON Biotechnology, Inc.) and centrifuged at 10,000 g for 10 min at 4°C. The protein concentration was determined using a protein assay with a bovine serum albumin standard. To detect PERK, IRE1α and ATF6 in mouse ovaries, sample buffer 6X [350 mM Tris-HCl (pH 6.8), 30% (w/v) glycerol (Kanto Chemical Co., Inc.), 0.012% (w/v) bromophenol blue (Kanto Chemical Co., Inc.), 6% (w/v) SDS and 30% (v/v) 2-mercaptoethanol (2-ME; Kanto Chemical Co., Inc.)] was added to each lysate, which was subsequently boiled at 95°C for 5 min and a total of 20 μg of proteins from the mouse ovaries were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Next, proteins were transferred onto Hybrid-polyvinylidene difluoride (PVDF) membranes (GE Healthcare Life Sciences, Chalfont, U.K.) using a Trans-Blot SD semi-dry electrophoretic transfer cell (catalog no. 1703940JA, Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) and a power supply with current set at 700 mA for 1 hr. After blocking with 5% Difco™ skim milk (catalog no. 232100; BD Biosciences, Franklin Lakes, NJ, U.S.A.) in Tris-buffered saline containing Tween-20 (TBST; 25 mM Tris/HCl, pH 8.0, 125 mM NaCl and 0.05% Tween-20), PVDF membranes were then incubated with primary antibody overnight at 4°C with anti-PERK, IRE1α and ATF6 (1:5,000, Cell Signaling Technology, Danvers, MA, U.S.A.) according to the manufacturer's recommendation and the PVDF membranes were washed three times (10 min each). Following extensive washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:3,000; Abcam, Cambridge, U.K.) for 1hr. After washing three times (10 min each) with TBST at room temperature, the immunoreactivity was visualized by enhanced chemiluminescence using the West-Q Chemiluminescent Substrate kit (GenDEPOT, Houston, TX, U.S.A.). The loading and blotting of the amount of protein was verified by reprobing the membrane with anti-β actin antibody (1:5,000; Abcam) followed by Coomassie Blue staining. Membrane images were digitized using the Imaging System of FUSION-Solo (6x, Vilber Lourmat, Marne La Vallée, France). The relative protein expression was obtained by comparing the respective specific band to the β actin control from the same membrane.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, U.S.A.). The mean ± SEM of the data were calculated. Student's *t*-tests were used to determine significance between two groups. One-way analysis of variance (ANOVA) with Dunn's Multiple Comparison Test was used to determine significant differences between three groups. An unpaired Student's *t*-test was used to compare the data between two groups. A P value <0.05 was considered to be statistically significant.

RESULTS

Effect of age on ovarian function and embryo development

An average of 29.92 oocytes were collected from 2-month-old mice following ovulation induction by treatment with hormones. In contrast, an average of only 19.0 and 18.9 oocytes could be collected from mice at 4 and 6 months of age. The cleavage rate after parthenogenetic activation of oocytes was significantly decreased with aging (P<0.05). Blastocyst development rates at 4 and 6 months of age, respectively, were also decreased compared to embryos derived from oocytes of 2-month-old mice. However, there was no significant difference between 4- and 6-month-old mice. A significant difference in total cell numbers in blastocysts was only present for mice aged 2 and 6 months (Table 1).

Age-dependent expression of genes related to inflammatory response and follicle development

We assayed mRNA expression of genes for inflammatory factors that are considered to be 'proinflammatory' and 'anti-inflammatory' in the mouse ovary. The relative expression of pro-inflammatory factor related gene *Tnfα* was significantly increased

with aging. *Il-6* and *Il-8* also increased with aging. In particular, the expression of *Il-6* was significantly increased in 6-month-old mice compared to mice that were 2 and 4 months old. In the case of *Il-8*, its expression in 2-month-old mice was lower compared to 4- and 6-month-old mice. The relative expression of the anti-inflammatory factor *Tgfβ* was highest in the ovaries of 6-month-old mice (Fig. 1a).

Nobox expression was decreased in aged mice, showing significant differences between 2- and 6-month-old mice. However, *Nano3* and *Lhx8* expression did not show significant differences with aging (Fig. 1b).

Aging of mice and upregulation of endoplasmic reticulum stress and apoptosis in the ovary

We examined the expression of ER stress and apoptosis genes in the ovaries of aged mice. Figure 2 shows the expression of genes in ovaries of 2-, 4- and 6-month-old mice. As age increased, expression at the mRNA level of ER stress and apoptosis related genes increased in the ovary.

The apoptosis related genes, *Bax* and *Bcl2*, did not show significant changes in expression depending on age, but the pro-proliferative gene, *c-Myc*, showed noticeably higher levels of mRNA expression in 6-month-old mice compared to 2-month-old mice (Fig. 2a).

The relative expressions of *X-box-binding protein* (*Xbps*)*, activating transcription factor 4* (*Atf4*) and *activating transcription factor 6α* (*Atf6*) increased significantly as mice got older. Meanwhile, *inositol requiring enzyme 1α* (*Ire1*) expression increased significantly in 4-month-old mice compared to 2-month-old mice, but not in 6-month-old mice. The 6-month-old mice showed significantly increased expression of *pancreatic ER kinase* (*Perk*) compared to 4-month-old mice, whereas 2-month-old mice did not show a difference (Fig. 2b).

Human EPCs restore the embryo development of aged mice

The next question was whether intravenous injections of hEPCs was able to rescue reduced age-related fertility. The fertility of mice can be evaluated by embryo development. Although the effect of hEPCs from different donors was investigated, it had no significant effect within each age group (data not shown). The average number of oocytes ovulated from mice treated with hEPCs did not differ from those of the same aged group of mice (Table 2). The number of fragmented or lysed oocytes was also not significantly affected by hEPCs treatment in mice. The *in vitro* development of oocytes collected from individual mice showed that mice given hEPCs produced more embryo cleavage and blastocysts with increased cell numbers compared with those without hEPCs treatment in the 4 months old groups (Table 2).

Intravenous hEPCs injection may influence inflammatory and follicle development gene expressions in ovaries

We injected human EPCs into mice intravenously, then examined the relative expressions of genes in ovaries. Among inflammation-related genes, expression of *Ifn-γ* and *Il-1β* decreased with hEPC treatment, both in 4- and 6-month-old mice. However, the relative expression of *Tnfα* after hEPC treatment decreased only in 6-month-old mice. The relative expression of *Il-8*, *Tgfβ* and *Il-10* did not show significant changes after hEPCs treatment (Fig. 3a).

The follicle related gene, *Nobox*, showed a significant increase in relative expression of mRNA after hEPC treatment, both in 4- and 6-month-old mouse ovaries. *Nano3* did not show a significant change in relative expression, while *Lhx8* expression was decreased in 4-month-old mice and increased in 6-month-old mice after hEPCs injection (Fig. 3b).

Human EPCs down-regulated apoptosis and ER stress related factors in ovaries

Between the two apoptosis related genes we examined, only *Bcl2* showed a significant increase of mRNA expression in both 4- and 6-month-old mice after intravenous hEPCs injection. The relative expression of *Bax* did not show noticeable changes. The pro-proliferative gene, *c-Myc*, also did not show a significant change in expression related to treatment with hEPCs (Fig. 4a).

Next, we examined the occurrence of ER stress in mouse ovaries treated with hEPCs by analyzing expression of an ER stress marker. After treatment of mice with human EPCs, the relative expression of *Ire1* was significantly downregulated in 4-month-old mice, but there was no significant difference in 6-month-old mice. The relative expression of *Atf4* was decreased after hEPCs treatment in 6-month-old mice while 4-month-old mice did not show a significant decrease related to hEPCs treatment. Expression levels of *Xbps*, *Perk*, and *Atf6* did not show significant differences related to hEPC treatment, either in 4-month-old or 6-month-old mice (Fig. 4b).

hEPCs modulated the expression of protein PERK, IRE1α and ATF6

Western blot was used to show the expression of PERK, IRE1α and ATF6 in mouse ovaries. The expression of PERK and IRE1α in 4- and 6-month-old mice treated with hEPCs was significantly reduced compared with the control group ($P<0.05$). However, there was no significant difference in ATF6 protein expression between the hEPCs treated and control groups (Fig. 5).

DISCUSSION

In the present study, we showed that with aging, the relative expression of pro-inflammatory cytokines including *Tnfα, Il-6* and *Il-8* as well as the pro-oncogene *c-Myc* increased. In addition, ER expression of stress-related genes including Atf6, Atf4 and Xbps in mouse ovaries progressively increased with aging. However, exogenous hEPCs protected against reduction of reproductive aging in mice as indicated by improved *in vitro* development as measured by cleavage rates, blastocyst formation rates and blastocyst cell

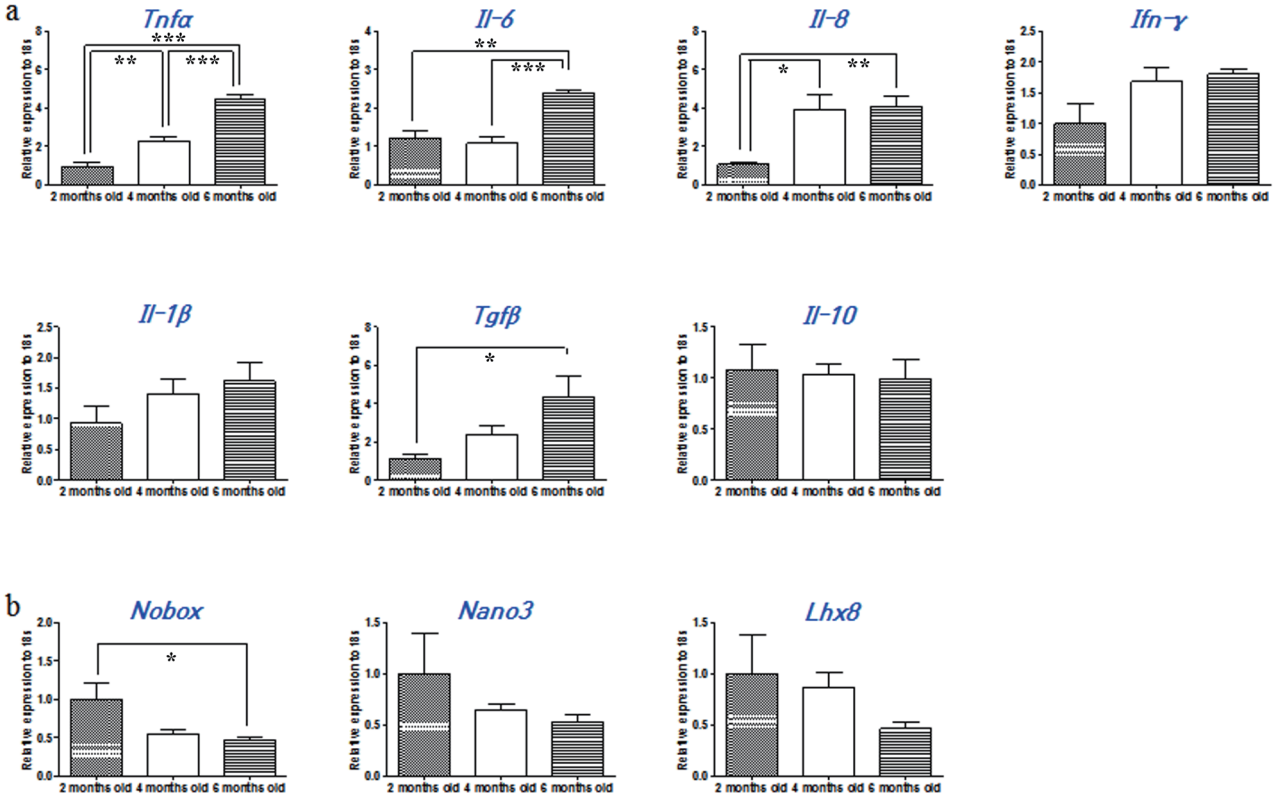

Fig. 1. Relative expression of inflammation and follicle development genes of mouse ovaries according to age. The relative mRNA expression levels of pro-inflammatory cytokine (*Tnfα*, *Il-6*, *Il-8*, *Ifn-γ* and *Il-1β*) and anti-inflammatory cytokines (*Tgfβ*, *Il-10*) (a) and follicle development related genes (*Nobox*, *Nano3* and *Lhx8*) (b) are shown. Significance at **P*<0.05, ***P*<0.01, ****P*<0.001. Data represent mean ± SEM. Replication was performed at least 6 times per group.

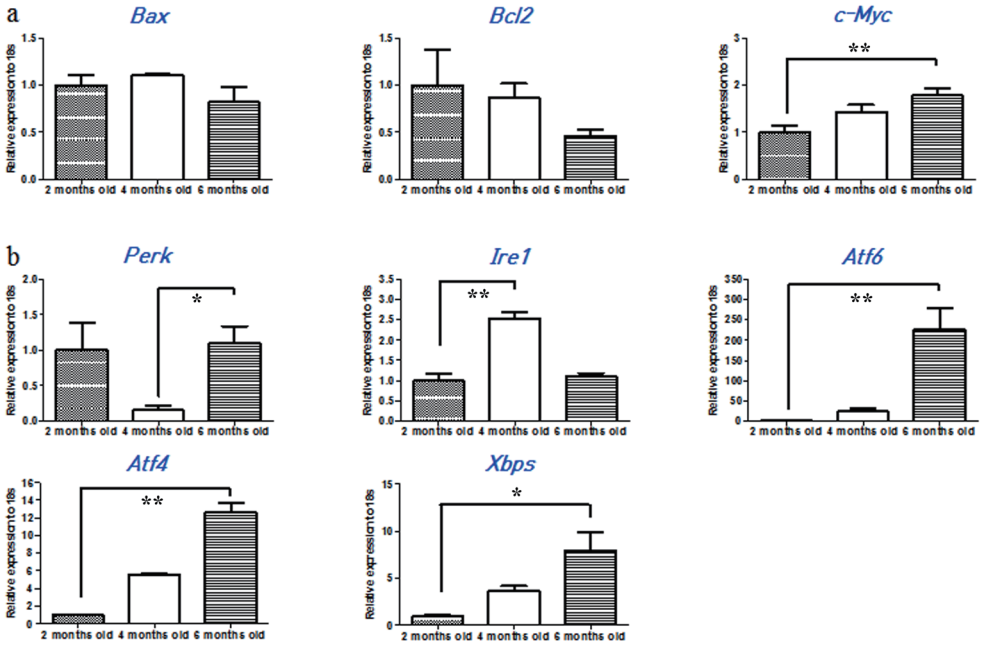

Fig. 2. Analysis of age-dependent gene expression related to apoptosis and endoplasmic reticulum (ER) stress in mouse ovaries. The mRNA expression levels of apoptosis (*Bax*, *Bcl2*, *c-Myc*) (a) and ER stress genes (*Perk*, *Ire1*, *Atf6*, *Atf4* and *Xbps*) (b) in 2 months old, 4 months old and 6 months old groups. Significance at **P*<0.05, ***P*<0.01. Data represent mean ± SEM. Replication was performed at least 6 times per group.

Table 2. Effect of intravenous hEPCs administration on ovulation and embryo development

	No. ovulated oocytes (Mean ± SEM, per mice)	*In vitro* development		Total cell no.
		No. cleaved embryos	No. blastocyst	
4 mo	257 (23.36 ± 4.15)	189 (68.82 ± 4.12)$^{ab)}$	57 (18.19 ± 3.58)$^{ab)}$	70.33 ± 7.34$^{ab)}$
4 mo EPCs	232 (21.09 ± 1.60)	188 (80.98 ± 2.19)$^{ab)}$	69 (30.66 ± 4.70)$^{c)}$	95.67 ± 2.47$^{ab)}$
6 mo	214 (19.45 ± 3.18)	78 (37.40 ± 5.78)$^{c)}$	21 (10.49 ± 2.36)$^{ab)}$	45.09 ± 3.69$^{b)}$
6 mo EPCs	232 (21.09 ± 1.97)	174 (73.58 ± 2.15)$^{bc)}$	39 (16.94 ± 1.94)$^{bc)}$	74.18 ± 2.32$^{bc)}$

Total of 44 ICR mice at 4 months and 6 months old, respectively were stimulated with PMSG and hCG. Results are presented as the mean ± SEM of 11 mice per group. Different superscripts within the same column represent significant differences (P<0.05) among groups. 4 mo old; 4-month-old mice group, 6 mo old; 6-month-old mice group.

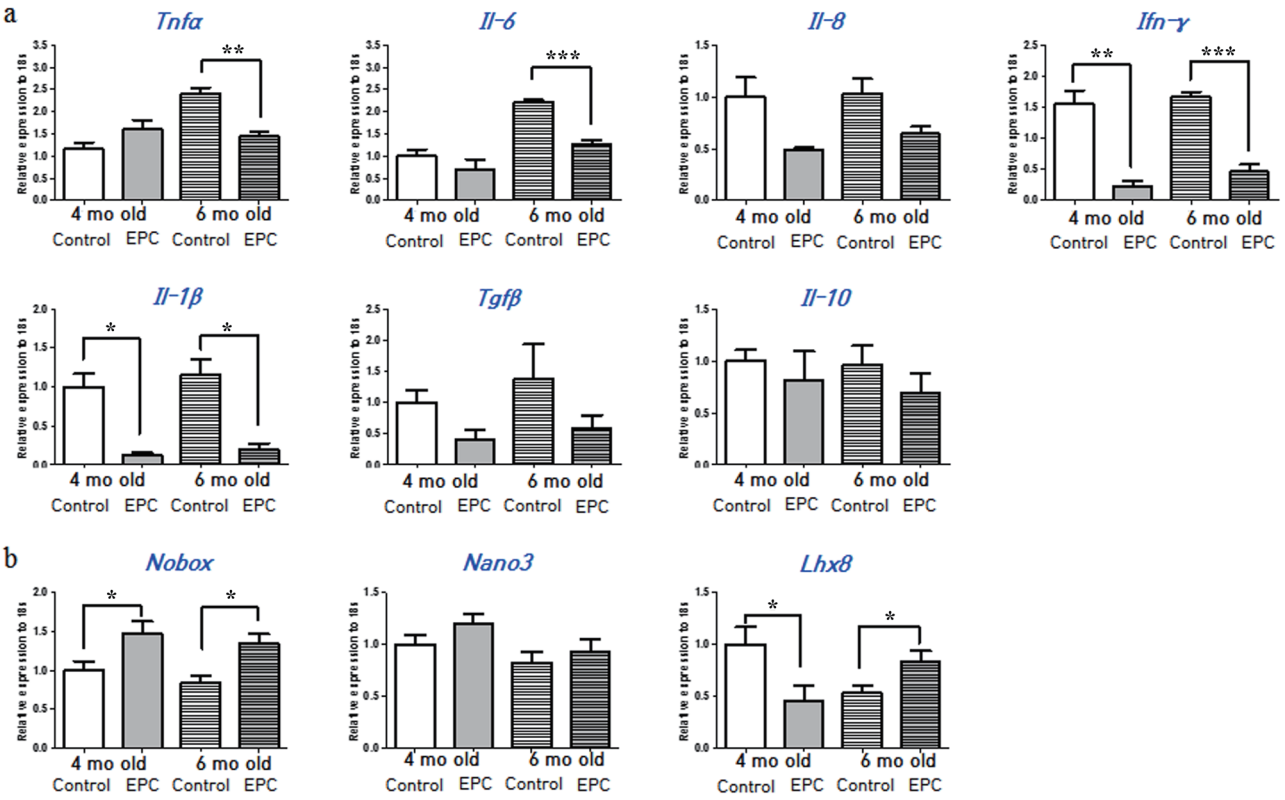

Fig. 3. hEPCs administration in mice altered the expression of inflammation and follicle development genes of mouse ovaries. The mRNA expression levels of pro-inflammatory cytokines (*Tnfα*, *Il-6*, *Il-8*, *Ifn-γ* and *Il-1β*) and anti-inflammatory cytokines (*Tgfβ*, *Il-10*) (a) and follicle development related genes (*Nobox*, *Nano3* and *Lhx8*) (b) in control and hEPCs in the 4 months old and 6 months old groups. 18s was used as the endogenous control. Significance at **P*<0.05, ***P*<0.01, ****P*<0.001. The mRNA levels are displayed as fold change and the data represent mean ± SEM, expressed by error bars. Replication was performed at least 6 times per group. 4 mo old; 4-month-old mice group, 6 mo old; 6-month-old mice group.

numbers. Furthermore, hEPCs improved levels of anti-inflammatory cytokines and reduced ER stress factors with respect to mRNA levels and protein expression of PERK and IRE1α.

In mammals, it is a well-established paradigm that females are born with a fixed number of oocytes that continually decline until few or none remain [12]. In our study, a significant reduction with aging was observed only in the quality of oocytes. Although reduced numbers of ovulated oocytes with aging were found, the number of ovulated oocytes was not affected by hEPCs injection. Failure to fully rescue ovarian aging by hEPCs could be related to exhaustion of existing germ cells regardless of treatment with hEPCs. In the present study, using a mouse model of age-associated infertility, intravenous administration of human EPCs had a beneficial effect on *in vitro* development of parthenogenetically activated oocytes. Therefore, it should be noted that hEPCs cannot rescue loss of the oocyte pool with aging but can significantly delay the age-related decline in fertility. It is likely that the beneficial effects of hEPCs on oocyte quality act via increased anti-inflammatory and decreased ER stress factors of hEPCs.

Cytokine functions in the ovary have been described as promoting the processes of follicular growth, steroidogenesis, follicle recruitment and activation of leukocytes which are all necessary for ovulation and tissue remodeling prior to and during ovulation

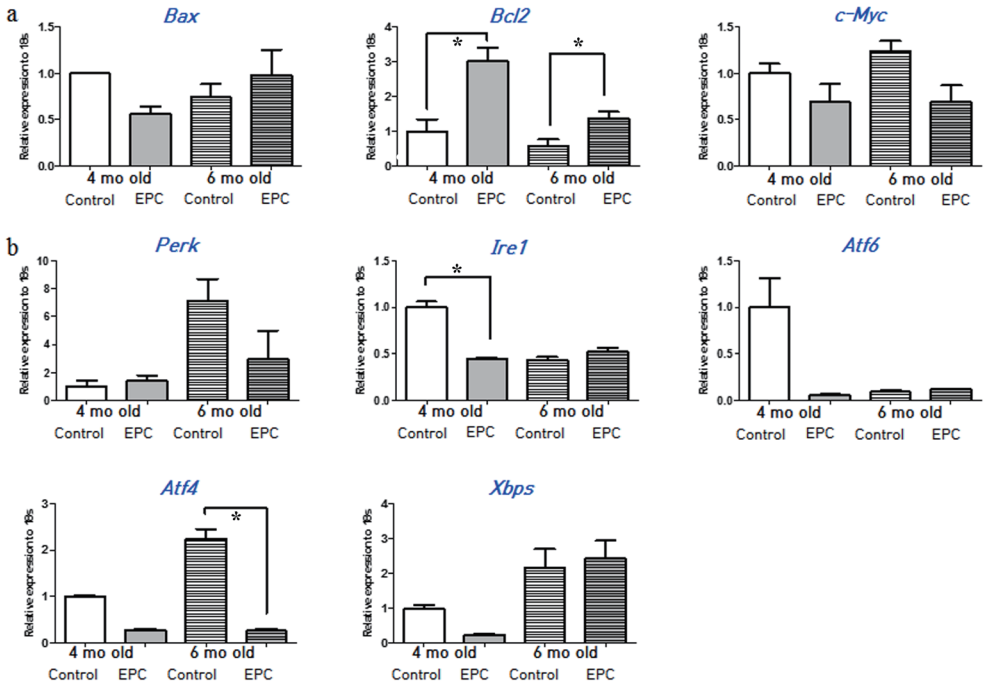

Fig. 4. Evaluation of apoptosis and endoplasmic reticulum (ER) stress changes in mouse ovaries. The mRNA expression levels of apoptosis (*Bax*, *Bcl2*, *c-Myc*) (a) and ER stress genes (*Perk*, *Ire1*, *Atf6*, *Atf4* and *Xbps*) (b) in control and hEPCs in the 4 months old and 6 months old groups. An increase in *Bcl2* expression level was observed in the hEPCs treated group compared with the control group. A significant decrease of Ire1 in 4-month-old mice and Atf4 in 6-month-old mice was observed after hEPCs treatment. Significance is set at **P*<0.05. The mRNA levels are displayed as fold change and the data represent mean ± SEM expressed by error bars. Replication was performed at least 6 times per group. 4 mo old; 4-month-old mice, 6 mo old; 6-month-old mice.

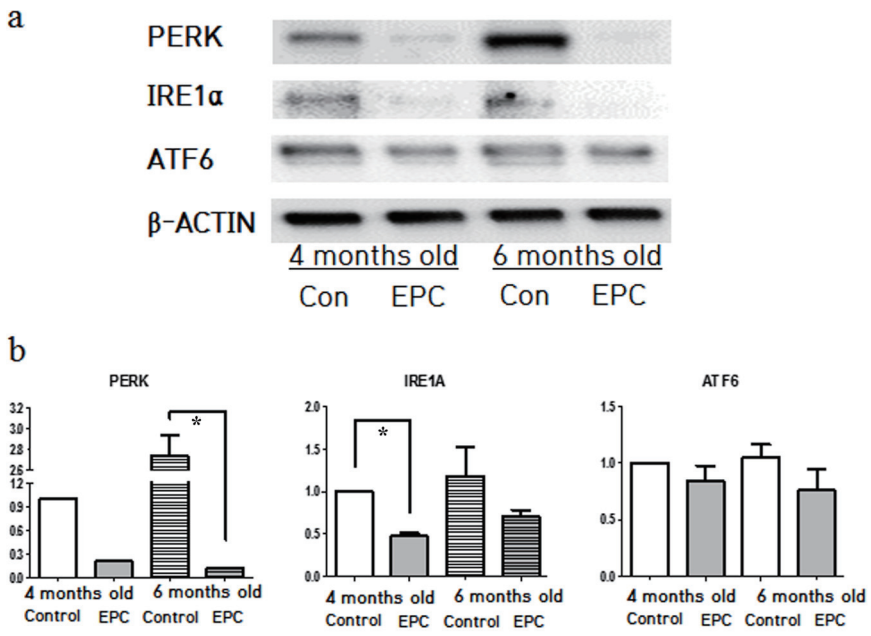

Fig. 5. Protein expression levels of PERK, IRE1α and ATF6 in ovaries of aged mice. Representative western blot images (a) and quantification of PERK, IRE1α and ATF6 protein expression levels (b). β-ACTIN served as an internal control. Data are expressed as the mean ± SEM, expressed by error bars.

[5]. Our study demonstrated that EPCs modulated levels of the inflammatory cytokines *Tnfα* and *Il-1β* which may indirectly account for the improvement in embryo development from oocytes of aged ovaries. Increased levels of follicular fluid (FF) TNFα

in women with polycystic ovarian syndrome are significantly and inversely correlated to FF E2 levels, which is again indicative of poor-quality oocytes and embryos [15, 36]. In our study, ovarian *Tnfα* increased with aging. The hEPCs injections may decrease Tnfα expression to counteract oocyte quality with aging. Therefore, we can conclude that ovarian *Tnfα* might deteriorate the microenvironment within the follicle, thereby negatively affecting oocyte and subsequent embryo quality [37]. It has also been suggested that *Ifn-γ* levels that exceed normal physiologic concentrations may inhibit ovulation and contribute to early pregnancy loss [28]. Although in the present study there were no differences in expression of *Ifn-γ* with aging, its expression was also decreased by hEPCs injection.

It has been recognized that ER stress contributes to apoptosis and tissue damage by downregulating anti-apoptotic *Bcl-2* [8]. In our study, *Bcl-2* expression showed no significant differences among the age groups of control mice, but hEPCs injections upregulated its expression. It has been suggested that the *c-Myc* gene is a direct downstream target of the estrogen receptor [2]. The increase in *c-Myc* expression may be due to a DNA damage response rather than a direct response to lowering of estrogen with aging. Misfolded and unfolded proteins accumulate in the ER during stress, resulting in initiation of the unfolded protein response (UPR) to alleviate side effects from ER stress and promote cell survival [21, 38]. The UPR is initiated to relieve the ER load through three pathways: (1) PERK/ATF4 ; (2) IRElα/XBP-1; and (3) ATF6α.

PERK is a single-pass ER transmembrane kinase whose activation paradoxically promotes the transcriptional initiation of ATF4. IRE1 is the most conserved ER stress sensor with both a Ser/Thr kinase domain and an endoribonuclease domain in its cytosolic portion. Furthermore, IRE1 is linked to the activation of inflammatory and apoptotic signaling [25, 34]. In addition, the kinase domain of IRE1α integrates ER stress with pro-inflammatory responses through direct binding with adaptor protein TNFα receptor associated factor 2 and subsequent activation of the nuclear factor-kappaB (NF-κB) and c-Jun N-terminal kinase (JNK) pathways [7, 18] which may play crucial roles in inflammatory responses [24]. In our study, the relative mRNA expression of *Ire1* and *Atf4* was significantly decreased after hEPCs treatment in 4- and 6-month-old mice, respectively. At the protein expression level, expression of Ire1α in 4-month-old mice was decreased and PERK also showed a significantly decreased profile in 6-month-old mice. Thus, protection against oxidative stress by EPCs could directly contribute to reduction of ER stress in aged mouse ovaries. The observed effects of hEPCs injection are all consistent with the notion that hEPCs may interactively drive the progression of competent oocyte development in the ovaries of aged mice.

hEPCs were isolated from peripheral blood, and have been shown to play an important role in tissue repair by exerting anti-inflammatory [31] or antioxidant actions [35, 39]. Consistent with the above findings, in our study hEPCs robustly increased the expression of anti-inflammatory genes coincident with decreased expression of ER stress at the mRNA and protein levels. Although intravenous injection of hEPCs cannot prevent age-associated infertility, it could delay reproductive aging.

There are obvious limitations in studying a single dose and inter-injection interval of EPCs in the present study. Additional studies are needed to determine the optimal dose and timing of cell administration. Nevertheless, short-term intravenous treatment with hEPCs for improving ovarian function and oocyte quality appears to sustain fertility in the aged mouse model.

Taken together, our data demonstrated that hEPCs supported maintenance of ovarian function and improved oocyte quality in aged mice. Our results suggest a potential mechanism for hEPCs therapy in age-associated infertility. EPCs effectively suppresses inflammatory cytokines, ER stress and apoptosis, thereby maintaining reproductive functions. Therefore, hEPCs represent not only a novel strategy for age-associated infertility but also may be useful for regenerative medicine and clinical applications.

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