

# Differences in the reaction of hyperlipidemia on different endothelial progenitor cells based on sex

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**Abstract.** The sex of a patient can affect the outcomes of several cardiovascular diseases, and men generally tend to experience earlier episodes of cardiovascular diseases compared with women. The progression of atherosclerosis during hyperlipidemia can be induced by reactive oxygen species (ROS) and oxidized-low-density lipoprotein (ox-LDL). By contrast, bone marrow (BM)-derived endothelial progenitor cells (EPCs) have been reported to serve a protective role against atherosclerosis. The aim of the present was to compare the effects of sex under conditions of hyperlipidemia on different populations of EPCs, and to identify the potential underlying mechanisms. EPC numbers and ROS levels in the blood and BM were measured using fluorescence activated cell sorting in male and female LDL receptor knock-out C57BL/6 mice maintained on a high-fat diet for 6 months, and in male and female wild type C57BL/6 mice following ox-LDL injection for 3 days. Female hyperlipidemic mice exhibited lower levels of plasma lipids, atherosclerotic plaque formation, intracellular EPC ROS formation and inflammatory cytokine

levels. Furthermore, BM CD34<sup>+</sup>/fetal liver kinase-1 (Flk-1<sup>+</sup>), CD34<sup>+</sup>/CD133<sup>+</sup> and stem cell antigen-1/Flk-1<sup>+</sup>, as well as all circulating EPCs, were maintained at higher levels in female hyperlipidemic mice. In addition, similar changes with regards to BM CD34<sup>+</sup>/Flk-1<sup>+</sup>, CD34<sup>+</sup>/CD133<sup>+</sup>, c-Kit<sup>+</sup>/CD31<sup>+</sup> and circulating CD34<sup>+</sup>/Flk1<sup>+</sup> and CD34<sup>+</sup>/CD133<sup>+</sup> EPCs were observed in female mice following ox-LDL treatment. These sustained higher levels of BM and circulating EPCs in female mice with hyperlipidemia may be associated with reduced levels of ox-LDL as a result of reduced intracellular ROS formation in EPCs and decreased inflammatory cytokine production.

## Introduction

Differences in the clinicopathological characteristics between men and women can be observed in a variety of diseases, including cardiovascular diseases (1-3). Women of reproductive age tend to be at lower risk of atherosclerosis, myocardial infarction and coronary artery disease (CAD) compared with men in the same age bracket and menopausal women (1). However, the mechanisms underlying these difference remain unknown.

Endothelial injury or dysfunction is considered to be a leading factor underlying the progression of atherosclerosis and CAD (4). Endothelial progenitor cells (EPCs) serve a key role in vascular re-endothelialization and angiogenesis, where they can suppress neointima formation after vascular injury (4). However, the effects of hyperlipidemia on the population profile of EPCs in different sexes and the related mechanisms remain poorly understood.

Oxidized-low-density lipoprotein (ox-LDL) has been reported to be a pivotal element in the hyperlipidemic status, where it has been previously observed to contribute to atherosclerotic plaque formation (5). Patients diagnosed with stable cardiovascular disease and acute coronary syndrome tend to

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exhibit higher levels of ox-LDL in the serum (6). It was also previously suggested that native LDL can be continuously converted to ox-LDL in the blood (7). Therefore, the levels of native LDL are closely associated with the levels of ox-LDL *in vivo* (7). Ox-LDL can inhibit the proliferation and differentiation of EPCs, thereby suppressing EPC migration, adhesion and vasculogenesis *in vitro*, and neovascularization after ischemia *in vivo* (8-10). In addition, a previous study suggested that treating wild type (WT) mice with human ox-LDL confers comparable effects as that of hyperlipidemia on EPCs in LDL receptor knock out (LDLR<sup>-/-</sup>) mice *in vivo* (11). However, the concentration of ox-LDL in serum differs between men and women (12). In addition, EPC numbers have also been reported to be higher in women of reproductive age compared with those in age-matched men and postmenopausal women (13,14). These previous observations suggest that EPC numbers may also differ between men and women with hyperlipidemia due to the differences in concentrations of ox-LDL and native LDL between the sexes.

Oxidative stress as a result of reactive oxygen species (ROS) production is an important mediator of atherosclerosis (15). It has been reported that ROS can be induced by elevated ox-LDL levels and a hyperlipidemic status (11,16). A previous study also showed that ROS can facilitate the conversion of native LDL to ox-LDL in WT mice in circulation (7). ROS levels have been demonstrated to be significantly higher in male rat cardiomyocytes, male human serum and vascular cells compared with those in women (17-19). Furthermore, both experimental and clinical results potentially suggest a more powerful antioxidant capacity in women compared with that in men (20). Proinflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , were previously found to be significantly increased in patients with hyperlipidemia (10) or in WT mice following ox-LDL treatment (21). These cytokines also promote hematopoietic cell development and function (22). Nevertheless, estrogen may act on estrogen receptors on EPCs to suppress the expression of genes related to pro-atherosclerosis, whilst promoting the expression of anti-atherosclerosis genes to downregulate proinflammatory cytokine expression (23).

The present study investigated atherosclerotic plaque formation and the numbers of bone marrow (BM) and circulating EPCs in female hyperlipidemic mice or following ox-LDL treatment. The aim was to explore the effects of hyperlipidemia and ox-LDL on EPCs in different sexes and investigate the underlying mechanisms.

## Materials and methods

**Preparation of ox-LDL.** All human procedures were performed in accordance with the Guidelines of the Human Research Ethics Committee of the Shandong Second Provincial General Hospital Affiliated to Shandong University (Jinan, China). The Human Research Ethics Committee of the Shandong Second Provincial General Hospital Affiliated to Shandong University (Jinan, China) approved the experimental protocols (approval no. XYK20181224). All participants agreed to use their samples for scientific research, and informed consent was obtained. In accordance with the Institutional Review Board under Food and Drug Administration regulations (24), venous blood was collected via puncturing the brachiocephalic vein

from 10 healthy male donors aged 21 to 32 years old, after they had provided consent, and the blood was collected in heparinized tubes on ice. Adults with diabetes, hyperlipidemia or other diseases that affect blood lipid levels were excluded. Lipoproteins were isolated from plasma using sequential ultracentrifugation with a Beckman TL-100 tabletop ultracentrifuge (Beckman Coulter, Inc.), which was extracted from blood supernatant by centrifuging at 1,500 x g for 20 min at 4°C (25). The lipoproteins were treated with 0.3 mM EDTA in 1X PBS (pH 7.4) overnight at 4°C and subsequently sterilized using a 0.22- $\mu$ M filter (MilliporeSigma). The Folin Lowry method was used to calculate the protein concentration in the lipoproteins. After dialysis using 5  $\mu$ M copper sulphate at 4°C overnight, ox-LDL was sampled from the native LDL immediately, as previously described (26). Thiobarbituric acid reactive substances (TBARS; Sigma-Aldrich; Merck KGaA) were used to monitor the degree of LDL oxidation and to ensure ox-LDL quality and reproducibility using a microplate reader at a wavelength of 532 nm (BioTek Instruments, Inc.) (27). Specifically, the TBARS value was maintained at 40-50 nmol malondialdehyde/mg protein. There were no detectable TBARS in the native LDL. All product was then stored at 4°C and used within 1 month of preparation.

**Animal model.** All animal procedures were performed in accordance with the Guidelines of the Animal Care Committee of the Shandong Second Provincial General Hospital Affiliated to Shandong University (Jinan, China). The Animal Care Committee of Shandong Second Provincial General Hospital Affiliated to Shandong University approved the experimental protocols (approval no. XYK20181225). All mice were maintained at room temperature with 40-60% humidity and a 12 h light/dark cycle, with *ad libitum* access to food and water.

A total of 10 randomized, age-matched wild-type (WT) male and female C57BL/6 mice (weight, 20 $\pm$ 3 g; age, 4-6 weeks; Jackson Laboratory) were administered 50  $\mu$ g prepared ox-LDL daily via tail vein injections for 3 days, as described previously (7). A total of 10 25 $\pm$ 5 g LDLR<sup>-/-</sup> C57BL/6 male and female mice (age, 4-6 weeks) were also obtained from Jackson Laboratory. The genotyping for WT and LDLR<sup>-/-</sup> mice were further confirmed by Southern blots. All mice were fed a normal diet (ND) until 8 weeks of age, after which they were fed a high-fat diet (HFD; 17% anhydrous milk fat and 0.2% cholesterol; Harlan Laboratories, Inc.) for 6 months to induce hyperlipidemia. Age-matched male and female WT C57BL/6 mice on an HFD or ND, and LDLR<sup>-/-</sup> male and female mice fed with ND were used as the controls.

After 6 months of HFD treatment, isoflurane was used to induce (3%) and maintain (1.5%) anesthesia in mice for blood collection (300-500  $\mu$ l) via cardiac puncture. Animals were then immediately euthanized using CO<sub>2</sub> (50-70% of the chamber volume per min) and death was confirmed by ascertaining cardiac and respiratory arrest or by observing fixed and dilated pupils. Aorta and BM were collected after confirming death of animals.

**Lipid profile measurements and atherosclerotic plaque ratio calculation.** After 6 months of HFD treatment, blood plasma samples from all mice were collected for lipid profile testing. Plasma (40  $\mu$ l) was tested using the Cholestech LDX lipid profile

cassette (Alere 10-989; Central Infusion Alliance, Inc.) for each test coupled with the Alere Cholestech LDX system (Alere Cholestech). Total cholesterol (TC), triglyceride (TRG), LDL, high density lipoprotein (HDL), non-HDL and the TC/HDL ratio were measured. Mouse aortas were also isolated for the atherosclerotic plaque formation test. Red oil (MilliporeSigma) was used to stain the atherosclerotic plaque at room temperature for 5 min, where the plaque area against the total inner surface of aorta was calculated as previously described (28).

**Analysis of EPCs.** BM and blood cells were harvested to observe the effects of ox-LDL and hyperlipidemia on the population of blood and BM EPCs in male and female mice. After eliminating red blood cells (RBCs) with RBC lysis buffer (Thermo Fisher Scientific, Inc.), a BD™ LSRII system (BD Biosciences) was used to perform multicolor analysis for BM and blood EPCs.

An endothelial cell marker combined with a stem cell marker, including CD34<sup>+</sup>/fetal liver kinase-1 (Flk-1)<sup>+</sup>, Stem cell antigen-1 (Sca-1)<sup>+</sup>/Flk-1, c-Kit<sup>+</sup>/CD31<sup>+</sup> and CD34<sup>+</sup>/CD133<sup>+</sup>, were used to identify EPCs as previously described (29). Functional EPCs express the endothelial markers Flk-1 and CD31 (29). BM and blood EPCs with a total of 50,000 cells in each sample were carefully analyzed and described (Fig. S1). All cell populations were carefully compensated (each cell population percentile was confirmed further using single antibody staining) and determined using flow cytometry, as previously described (30-36). Flk-1 APC-Cy™7 (cat. no. 561252) antibody was obtained from BD Biosciences and CD34 FITC (cat. no. 11-0341-82) from eBioscience (Thermo Fisher Scientific, Inc.). Sca-1 AF700 (cat. no. 108142), c-Kit-APC (cat. no. 105812), CD31-PE-Cy7 (cat. no. 102418) and CD133-PE (cat. no. 141204) were purchased from BioLegend, Inc. All antibodies were diluted 1:100.

**Intracellular ROS detection.** Mouse BM and blood were harvested following intravenous injection of 50 µg ox-LDL into each mouse for 3 days, as described previously (7). For LDLR<sup>-/-</sup> mice, BM and blood were harvested after 6 months of HFD feeding. RBC lysis buffer was used to remove all RBCs (37). A total of four groups of BM and circulating EPCs were selected for intracellular ROS detection. The mean of the four groups of ROS levels in EPCs were statistically analyzed.

Intracellular ROS generation was measured using FITC conjugated ROS Detection Reagent (cat. no. D399; Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (38). A total of 1x10<sup>6</sup> were incubated at 37°C for 10 min with 5 µg/ml reagent. All labeled cells were washed with PBS twice before suspending in warm PBS. Flow cytometry was used for analysis. BD™ LSRII (BD Biosciences) at a wavelength of 525 nm was used to calculate the positively fluorescent cells, as previously described (39).

**Measurement of proinflammatory cytokines.** Mouse blood samples were harvested after 6 months of HFD or ND treatment. The plasma was obtained from the blood samples after centrifugation at 300 x g for 20 min at 4°C. The plasma levels of the proinflammatory cytokines IL-1β (cat. no. 432601) and TNF-α (cat. no. 430904) were evaluated using ELISA kits from BioLegend, Inc. according to the manufacturer's protocols.

**Statistical analysis.** Data are presented as the mean ± standard deviation, and analyzed using an unpaired Student's t-test (two-sided) for comparisons between two groups of data, or a two-way ANOVA followed by a Bonferroni post hoc test for comparing the subgroups of data between male and female groups to minimize type I errors as appropriate in GraphPad Prism version 4 (GraphPad Software, Inc.). A two-tailed P<0.05 was considered to indicate a statistically significant difference.

## Results

**Lipid levels and atherosclerosis formation are lower in female mice with hyperlipidemia.** To study the effects of differences in sex on hyperlipidemia, male and female LDLR<sup>-/-</sup> mice were fed a HFD for 6 months. The TC, TRG, LDL and non-HDL lipoprotein levels, and the TC/HDL ratio were markedly increased in male and female hyperlipidemic LDLR<sup>-/-</sup> mice fed a HFD compared with their respective control groups, confirming that the hyperlipidemic mouse model was successfully established (Table I). Of note, the lipid levels in female hyperlipidemic mice was notably lower compared with that in male hyperlipidemic mice (Table I). In addition, a number of atherosclerotic plaques were present in the aorta of the male hyperlipidemic mice, whereas plaque severity was significantly decreased in the female mouse group (P<0.01; Fig. 1).

**BMEPC numbers are increased in female hyperlipidemic mice.** Persistent endothelial cell dysfunction or injury promotes the progression of atherosclerosis and coronary heart disease (4). Therefore, the EPC profiles were examined in the BM of male and female LDLR<sup>-/-</sup> mice. Hyperlipidemia did not change the BM Sca-1<sup>+</sup>/Flk-1<sup>+</sup>, c-Kit<sup>+</sup>/CD31<sup>+</sup> and CD34<sup>+</sup>/CD133 levels in male mice (Fig. 2B-D), which only exhibited significantly decreased CD34<sup>+</sup>/Flk-1<sup>+</sup> levels (P<0.05; Fig. 2A). By contrast, these BM EPC cell populations, except for those expressing c-Kit<sup>+</sup>/CD31<sup>+</sup>, were found to be significantly increased in female LDLR<sup>-/-</sup> mice fed a HFD compared with female LDLR<sup>-/-</sup> mice fed a ND (P<0.05) and those in male LDLR<sup>-/-</sup> mice fed a HFD (P<0.05; Fig. 2A, B and D). The c-Kit<sup>+</sup>/CD31<sup>+</sup> cell population in female hyperlipidemic mice was lower compared with that in the male mice, which may be due to the low basal numbers of this cell population in female mice (Fig. 2C).

**Blood EPC numbers are high in female hyperlipidemic mice.** The circulating EPC numbers were also measured in both LDLR<sup>-/-</sup> male and female mice. As shown in Fig. 2E-H, the numbers of circulating EPCs were significantly reduced in both male and female hyperlipidemic mice (P<0.05) compared with those in their control groups fed a ND. The numbers of blood EPCs, including those expressing CD34<sup>+</sup>/Flk-1<sup>+</sup>, Sca-1<sup>+</sup>/Flk-1<sup>+</sup>, c-Kit<sup>+</sup>/CD31<sup>+</sup> and CD34<sup>+</sup>/CD133<sup>+</sup>, were significantly higher (P<0.05) in the hyperlipidemic female mice compared with those in the respective male counterparts (Fig. 2E-H).

**Lower levels of blood intracellular ROS, plasma TNF-α and IL-6 are observed in female hyperlipidemic mice.** To investigate the cause of the differences in EPC numbers

Table I. Male and female mouse plasma lipid profiles.

Male, n=8 <sup>d</sup>	WT+ND	WT+HFD	KO+ND	KO+HFD
TC, mg±dl	104.7±3.6	207.3±34.2 <sup>b</sup>	231.5±24.4	1,713±215.8 <sup>a</sup>
HDL, mg±dl	56.7±12.9	102.1±4.4 <sup>b</sup>	77±8.3	74.4±22.8 <sup>a</sup>
TRG, mg±dl	93±33.3	158±12.4 <sup>b</sup>	102.3±28.7	594.3±203.5 <sup>a</sup>
LDL, mg±dl	5.4±4.1	12.5±1.3 <sup>b</sup>	134.8±19.6	1,586±90.1 <sup>a</sup>
Non-HDL, mg±dl	19.2±6.7	26.5±2.1 <sup>b</sup>	124.5±68.8	1,711±85.6 <sup>a</sup>
TC/HDL	1.2±0.1	2±0.4 <sup>b</sup>	2.8±0.4	23.3±4.9 <sup>a</sup>
Female, n=10 <sup>d</sup>	WT+ND	WT+HFD	KO+ND	KO+HFD
TC, mg±dl	101.2±2.4	185±24 <sup>b,c</sup>	113.7±9.3 <sup>c</sup>	775±94.8 <sup>a,c</sup>
HDL, mg±dl	68.6±6.5	76±10.1 <sup>c</sup>	74.3±2.5	73±12.7
TRG, mg±dl	89.4±10.2	49.9±7 <sup>b,c</sup>	61.7±5.7 <sup>c</sup>	508±65.1 <sup>a,c</sup>
LDL, mg±dl	4.5±2.1	10.4±2.5 <sup>b</sup>	26.7±7.2 <sup>c</sup>	596±101.8 <sup>a,c</sup>
Non-HDL, mg±dl	15.6±5.4	20.4±3.4 <sup>b,c</sup>	38.7±7.4 <sup>c</sup>	697±89.1 <sup>a,c</sup>
TC/HDL	1.5±0.4	2.4±1.1 <sup>b,c</sup>	1.5±0.1 <sup>c</sup>	10.6±7.5 <sup>a,c</sup>

<sup>a</sup>P<0.001 vs. respective KO+ND group; <sup>b</sup>P<0.001 vs. respective WT+ND group.; <sup>c</sup>P<0.001 vs. the respective male group. <sup>d</sup>Data are presented as the mean ± standard deviation. HDL, high density lipoprotein; LDL, low density lipoprotein; TRG, triglyceride; TC, total cholesterol; HFD, high fat diet; ND, normal diet; WT+ND, WT C57BL/6 mouse fed a ND for 6 months; WT+HFD, WT C57BL/6 mouse with HFD for 6 months; KO+ND, LDLR<sup>-/-</sup> mice with ND for 6 months; KO+HFD, LDL receptor knockout mice fed a HFD for 6 months.

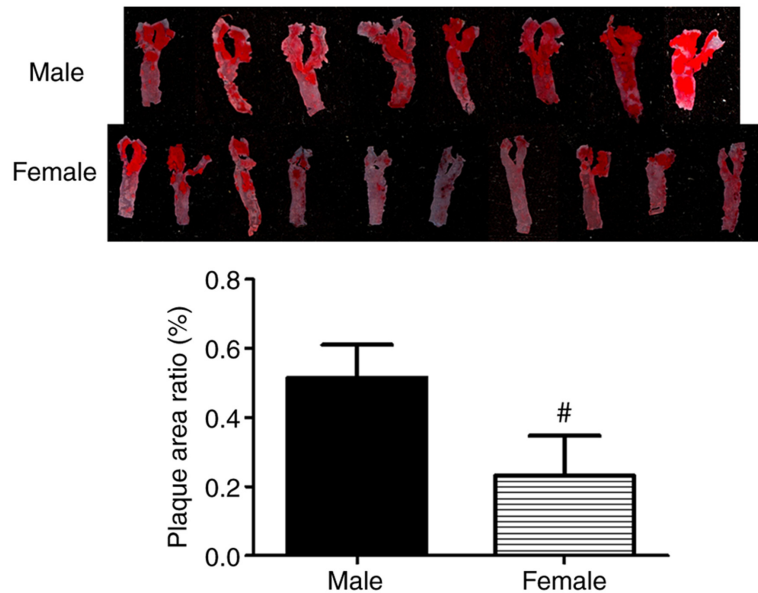


Figure 1. Mouse aortic atherosclerotic formation. Aortas were isolated and stained for atherosclerotic plaque analysis in the male and female hyperlipidemic LDLR<sup>-/-</sup> mice after 6 months of feeding with a high fat diet. A significant amount of atherosclerotic plaque was identified in the hyperlipidemic LDLR<sup>-/-</sup> mice. The levels of atherosclerotic lesions in the female hyperlipidemic LDLR<sup>-/-</sup> mice (lower panel; n=10) was significantly reduced compared with the male mice (upper panel; n=8). <sup>#</sup>P<0.001. LDLR<sup>-/-</sup>, low density lipoprotein receptor knock out.

between male and female mice with hyperlipidemia, the BM and blood EPC intracellular ROS levels were determined. Although there were no intracellular ROS changes in BM EPCs in both male and female mice, a significantly increased ROS level was observed in the blood EPCs of male hyperlipidemic mice compared with that in their corresponding control group fed a ND (P<0.01; Fig. 3A and B). The blood EPC intracellular ROS levels were significantly decreased in

female LDLR<sup>-/-</sup> mice fed with HFD compared with that in the female LDLR<sup>-/-</sup> mice fed with ND (P<0.05), and was also significantly lower compared with that in the male LDLR<sup>-/-</sup> mice fed a HFD (P<0.05; Fig. 3B).

The plasma inflammatory factor TNF- $\alpha$  and IL-6 levels were next measured in both male and female mice. After feeding the mice with a HFD for 6 months, except for those in the female WT mice, the TNF- $\alpha$  and IL-6 levels were



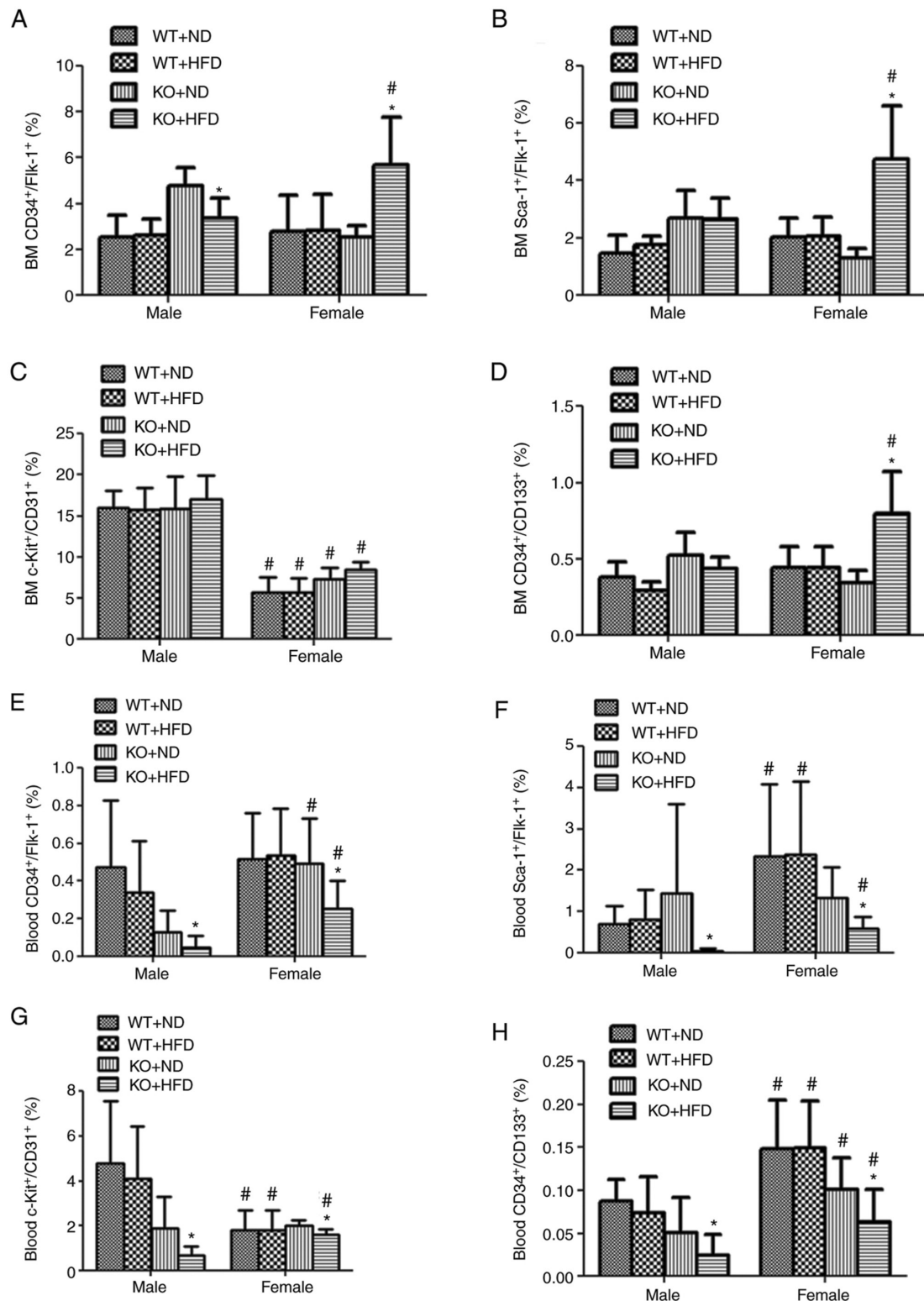


Figure 2. Sustained high level of murine BM and circulating EPCs in female hyperlipidemic mice. After 6 months of feeding with a HFD, the peripheral blood cells and BM were harvested, and the red blood cell were eliminated for flow cytometry analysis. (A-H) Populations of the cells expressing CD34<sup>+</sup>/Flk-1<sup>+</sup>, or Sca-1<sup>+</sup>/Flk-1, or c-Kit<sup>+</sup>/CD31<sup>+</sup> or CD34<sup>+</sup>/CD133<sup>+</sup> in both BM and blood were analyzed. The murine BM and circulating EPCs were maintained at a higher level in female hyperlipidemic mice compared with the males. \*P<0.05 vs. KO+ND; #P<0.05 vs. the respective male group. BM, bone-marrow; EPC, endothelial progenitor cell; HFD, high-fat diet; ND, normal diet; Flk-1, fetal liver kinase-1; Sca-1, stem cell antigen-1; LDLR<sup>-/-</sup>, low density lipoprotein receptor knock out; WT+ND, WT C57BL/6 mouse with ND for 6 months; WT+HFD, WT C57BL/6 mouse with HFD for 6 months; KO+ND, LDLR<sup>-/-</sup> mice with ND for 6 months; KO+HFD, LDLR<sup>-/-</sup> mice with HFD for 6 months.

found to be significantly increased in both female and male LDLR<sup>-/-</sup> mice compared with those in the LDLR<sup>-/-</sup> mice fed a ND (P<0.05; Fig. 4A and B). Of note, the TNF-α and IL-6

levels in all HFD-fed female LDLR<sup>-/-</sup> mice were significantly lower compared with those in the male LDLR<sup>-/-</sup> mice fed a HFD (P<0.05; Fig. 4A and B).

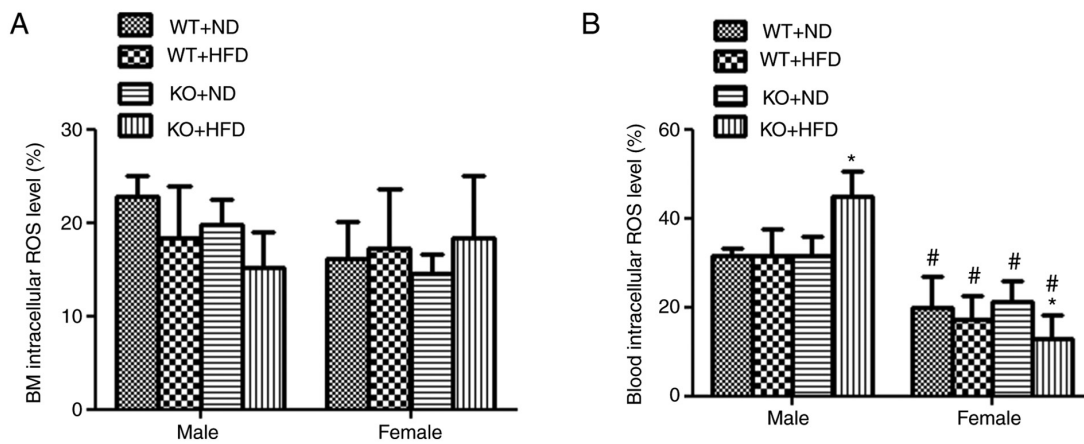


Figure 3. Decreased levels of intracellular ROS formation in blood EPCs in female hyperlipidemic mice. (A) There were no changes in intracellular ROS formation in the BM in all groups of mice. (B) Intracellular ROS production is significantly increased in the blood EPCs in the male hyperlipidemic LDLR<sup>-/-</sup> mice, whereas it decreased or was not changed in the female mice. \*P<0.01 vs. KO+ND; #P<0.01 vs. the respective male group. EPC, endothelial progenitor cell; ROS, reactive oxygen species; BM, bone marrow; ND, normal diet; LDLR<sup>-/-</sup>, low density lipoprotein receptor knock out; WT+ND, WT C57BL/6 mouse with ND for 6 months; WT+HFD, WT C57BL/6 mouse with HFD for 6 months; KO+ND, LDLR<sup>-/-</sup> mice with ND for 6 months; KO+HFD, LDLR<sup>-/-</sup> mice with HFD for 6 months.

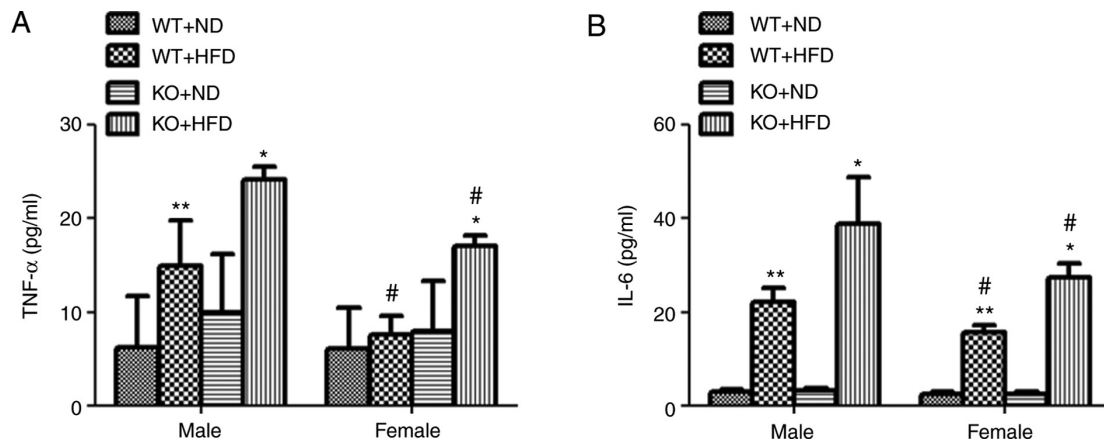


Figure 4. Low levels of proinflammatory cytokine secretion in female hyperlipidemic mice. There was a significant increase in (A) TNF- $\alpha$  and (B) IL-6 in male and female KO and male WT mice fed a HFD compared with the ND control group. However, the total levels of TNF- $\alpha$  and IL-6 in male mice were considerably higher than that in the female mice after HFD feeding. \*\*P<0.01, \*\*\*P<0.01 vs. KO+ND; #P<0.01 vs. the respective male group. LDLR<sup>-/-</sup>, low density lipoprotein receptor knock out; WT+ND, WT C57BL/6 mouse with ND for 6 months; WT+HFD, WT C57BL/6 mouse with HFD for 6 months; KO+ND, LDLR<sup>-/-</sup> mice with ND for 6 months; KO+HFD, LDLR<sup>-/-</sup> mice with a high fat diet for 6 months.

*High numbers of BM and circulating EPCs coupled with low levels of intracellular ROS are observed in female mice following ox-LDL treatment.* Ox-LDL treatment was used as the primary hyperlipidemic mediator to treat both male and female WT mice for 3 days prior to measuring their EPC numbers and intracellular ROS levels. In ox-LDL-treated male mice, the BM CD34<sup>+</sup>/Flk-1<sup>+</sup>, c-Kit<sup>+</sup>/CD31<sup>+</sup> (Fig. 5A and C) and circulating Sca-1<sup>+</sup>/Flk-1<sup>+</sup> and c-Kit<sup>+</sup>/CD31<sup>+</sup> (Fig. 5F and G) cell populations were significantly decreased (P<0.05) compared with mice without ox-LDL treatment. However, BM and circulating CD34<sup>+</sup>/CD133<sup>+</sup> (Fig. 5D and H) and circulating CD34<sup>+</sup>/Flk-1<sup>+</sup> cell numbers (Fig. 5E) were significantly increased (P<0.05). There was little to no change in the entire EPC population (Fig. 5A, B and D-H), except for the fact that the BM c-Kit<sup>+</sup>/CD31<sup>+</sup> population (Fig. 5C) was significantly increased (P<0.05) in female mice following ox-LDL treatment compared with female mice without ox-LDL treatment (Fig. 5). The female BM and

circulating CD34<sup>+</sup>/Flk-1<sup>+</sup> (Fig. 5A and E), CD34<sup>+</sup>/CD133<sup>+</sup> (Fig. 5D and H) and BM c-Kit<sup>+</sup>/CD31<sup>+</sup> (Fig. 5C) populations were significantly increased (P<0.05) compared with those in male mice treated with ox-LDL.

Subsequently, the BM and blood EPC intracellular ROS levels were measured. Similar to that in the hyperlipidemic mice, there were no changes in ROS levels in the BM in both male and female mice with or without ox-LDL treatment (Fig. 6A). However, the intracellular blood ROS levels were significantly elevated in ox-LDL-treated male mice (P<0.01) compared with that in the ox-LDL-treated female mice (Fig. 6B).

## Discussion

The present study demonstrated that the plasma lipid levels and atherosclerotic plaque formation were notably reduced in female hyperlipidemic mice. The BM and circulating EPCs

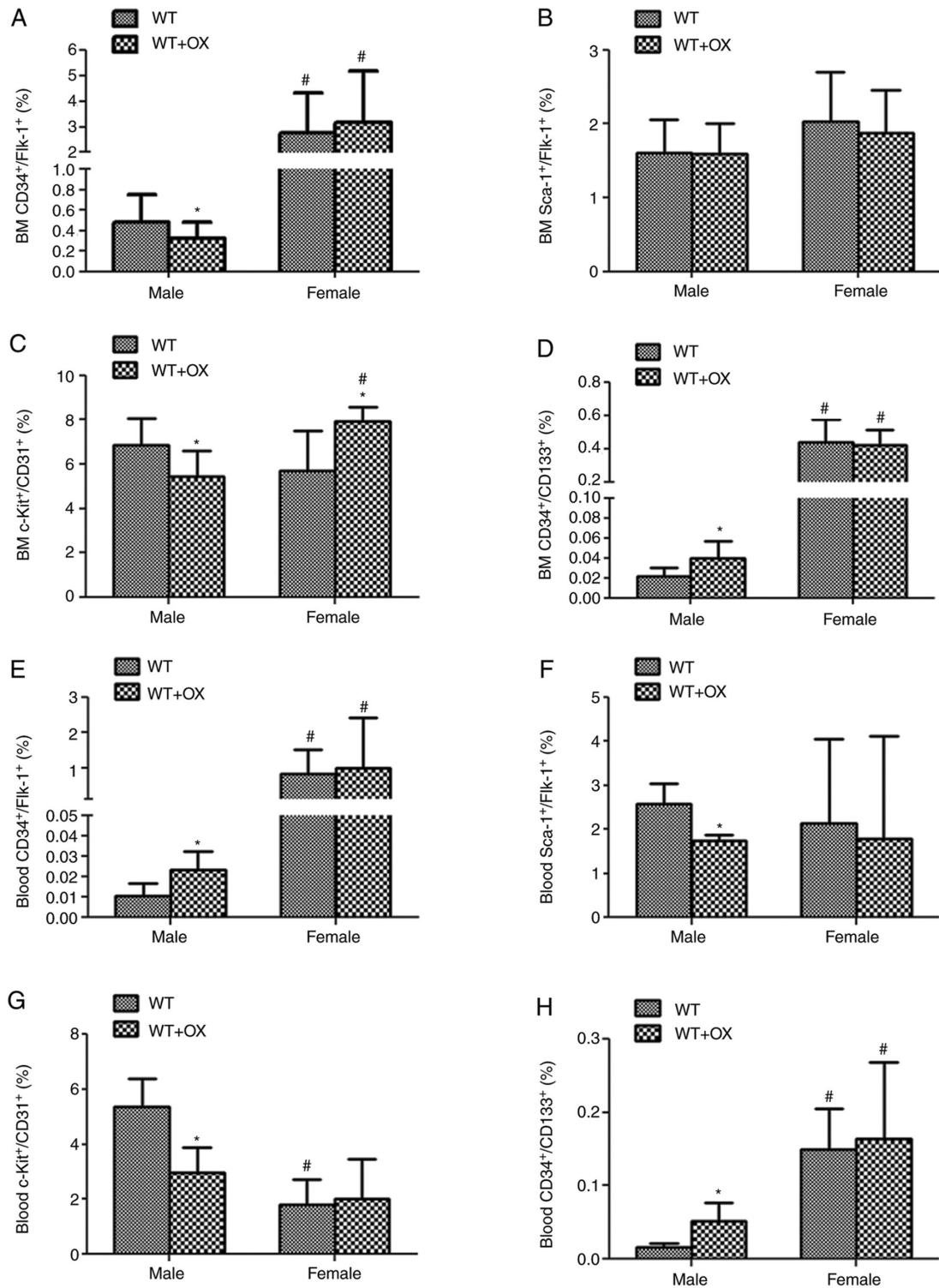


Figure 5. Sustained high levels of murine BM and circulating EPCs in female WT mice following ox-LDL treatment. (A-H) After 3 days of ox-LDL injection, the BM and peripheral blood cells were collected, and the red blood cells were eliminated for flow cytometry analysis. The EPC populations in both BM and blood were analyzed as shown. Following ox-LDL treatment, the murine BM and circulating EPCs were maintained at a higher level in female mice compared with the male mice. \*P<0.05 vs. WT+OX; #P<0.05 vs. the respective male group. ox-LDL, oxidized low-density lipoprotein; BM, bone marrow; EPC, epithelial progenitor cell.

were maintained in higher numbers in female mice with hyperlipidemia and following ox-LDL treatment. The potential mechanisms may be associated with lower levels of intracellular blood EPC ROS formation, and native LDL, plasma IL-6 and TNF- $\alpha$  levels in female mice compared with those in their male counterparts. To the best of our knowledge, the present

study was the first to investigate the effect of differences in sex on the reaction to hyperlipidemia and ox-LDL in different subgroups of EPCs in the BM and blood.

Ox-LDL is an important mediator of hyperlipidemia that is closely associated with a number of cardiovascular diseases (1-3). Ox-LDL interrupts the activity of EPCs

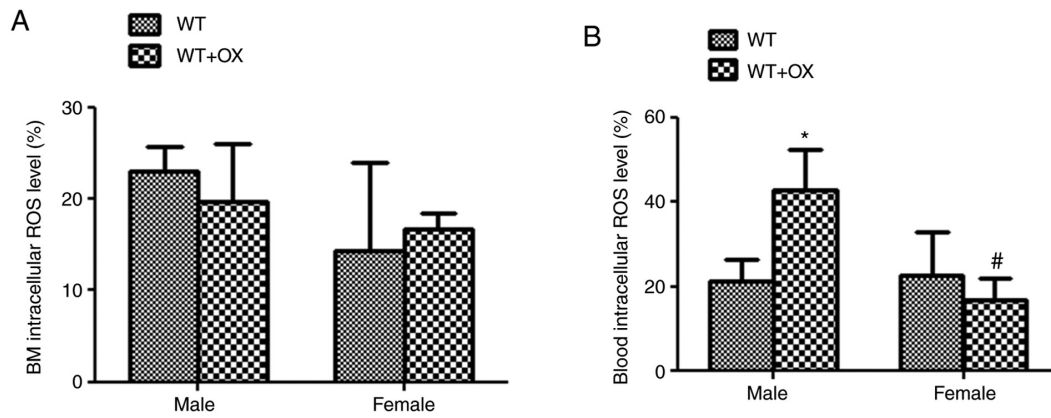


Figure 6. Intracellular ROS formation in blood EPCs in male mice is increased following ox-LDL treatment. (A) No changes in the intracellular ROS formation were observed in the BM in all groups of mice. (B) Intracellular ROS production is significantly increased in the blood EPCs in the male mice following ox-LDL treatment, while there were no changes in the female mice. \* $P < 0.01$  vs. WT+OX; # $P < 0.01$  vs. respective male group. EPC, endothelial progenitor cell; BM, bone marrow; ROS, reactive oxygen species; ox-LDL, oxidized low-density lipoprotein.

through various mechanisms, including the downregulation of E-selectin and integrin  $\alpha(v)\beta$  (5) expression, suppression of endothelial nitric oxide synthase, acceleration of cell senescence, suppressing telomerase, promotion of ROS generation and proinflammatory factor secretion in cardiovascular diseases, possibly due to the cardioprotective effects of estrogen (1). It has been reported that estrogen can inhibit EPC apoptosis and senescence, whilst promoting EPC mobilization (40). In addition, greater migratory activity and colony-forming capacity *in vitro* are also exhibited by EPCs isolated from middle-aged women compared with those isolated from men, which provides further support of the protective effects of endogenous estrogen on EPC function (41). In the present study, almost all groups of BM and blood EPCs were maintained at higher numbers in female mice with hyperlipidemia or following ox-LDL treatment compared with their male counterparts. Apart from the direct effects on EPC, estrogen may also provide a beneficial environment for EPCs, including suppression of pro-atherogenic gene expression, induction of atheroprotective gene expression, downregulation of IL-6 expression (42), generation of protective growth factors, including vascular endothelial growth factor and insulin-like growth factor 1 (43,44), in addition to the upregulation of suppressor proteins of cytokine signaling, resulting in resistance to the effects of deleterious TNF- $\alpha$  signaling in women (45,46). In support of these previous findings, the present study also demonstrated that native plasma LDL, IL-6 and TNF- $\alpha$  levels were considerably lower in female mice compared with those in males.

Although androgen receptors are expressed by EPCs, there is only limited evidence showing the effects of androgens on EPCs. Fadini *et al* (47) suggested that there is no correlation between androgen stimulation and late EPC expansion and adhesion *in vitro* after isolating both early and late human EPCs. Nevertheless, the number of circulating EPCs decreased after castration, and this reduction was irreversible, even with exogenous testosterone administration (47). In a previous clinical study of healthy middle-aged men, circulating EPCs were shown to exhibit a closer correlation with estrogen compared with testosterone (48). The growth-stimulatory and pro-survival effects of testosterone

may be limited to mature progenitor cells (48). In addition, analysis of plasma steroid levels in patients with irritable bowel syndrome found that EPCs were not correlated with testosterone levels (49). Therefore, testosterone may well be less influential than estrogen on EPC physiology, although conflicting evidence exists concerning the effects of androgens in this context.

The formation of ROS and the resulting oxidative stress are important mechanisms underlying the effects of ox-LDL (7). ROS can disrupt normal EPC function and is related to a variety of diseases, including CAD, diabetes, hyperlipidemia and renal ischemia-reperfusion injury (8-10,15). It has been reported that the extent of oxidative stress was higher in males than females in rats (17), human serum (18) and human vascular cells (19). In addition, both previous experimental and clinical studies suggested a potentially more powerful antioxidant capacity in females over males (20). The present study suggested a correlation between sex differences and the levels of oxidative stress, where females are less prone to ROS damage. A previous study on mice suggested that the stronger antioxidant protection from ROS in females may be related to the higher levels of pulmonary and brain superoxide dismutase (SOD) activity (50). Additionally, it has been demonstrated that estradiol can activate the MAP kinase signaling pathway, to upregulate manganese-SOD gene expression (51). Another study reported that estrogen can act as an antioxidant scavenger to eliminate free radicals due to the presence of the phenolic hydroxyl group (17). ROS levels were also shown to be higher in spayed female rats compared with corresponding female controls, but no significant difference was found in male rats after castration (17). By contrast, differences in the expression of NADPH-oxidase subunits were also observed between the two sexes, with higher expression of Nox1 and Nox4 in males compared with that in females (52). The higher expression of Nox1 and Nox4 in men could partially explain why males are more susceptible to oxidative stress than females. The present study showed that intracellular blood EPC ROS formation was reduced in female mice with hyperlipidemia or after ox-LDL treatment compared with that in their male counterparts.



There remain a number of questions on the mechanisms underlying the effects of hyperlipidemia or ox-LDL on EPCs in different sexes that need to be addressed. It is well-established that the identification and characterization of EPCs is a challenging and complex process as shown in previous studies (53-57). There are no uniform criteria for the identification of EPCs as of yet. However, combinations of a variety of cell markers are frequently used to characterize EPCs in the literature. Specifically, CD34<sup>+</sup>/Flk-1<sup>+</sup>, Sca-1<sup>+</sup>/Flk-1<sup>+</sup>, c-Kit<sup>+</sup>/CD31<sup>+</sup> and the CD34<sup>+</sup>/CD133<sup>+</sup> cell populations are primarily distributed in the blood and BM, and confer protective effects on the cardiovascular system (58-61). To provide a broader picture on the sex-specific reaction of EPCs under conditions of hyperlipidemia or after ox-LDL treatment, the specific EPC population responsible for the protective effects against atherosclerosis must be investigated in a future study. In addition, other potential mechanisms mediating the high numbers of BM and circulating EPCs in female mice warrants further study. The effect of estrogen on the modulation of blood lipid levels and estrogen receptors on EPCs upstream of plaque formation is a line of research worthy of further investigation. Furthermore, the application of these findings in clinical studies and the respective outcomes would be the ultimate desired outcomes of research into this field.

In conclusion, the present study demonstrated that the decreased atherosclerotic plaque formation in female mice with hyperlipidemia compared with male mice may be due to the sustained high numbers of BM and circulating EPCs in association with lower levels of intracellular blood EPC ROS formation, plasma TNF- $\alpha$  and IL-6 levels, and plasma native LDL levels in female mice compared with those in male mice.

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#### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

#### Authors' contributions

YC and LC designed the experiments. XZ, HB, YJ, XM, LY, ST, QZ, YX and YC performed the experiments. ZS, KH, LC,

PZ and HS collected and analyzed the data. YC and ST wrote the manuscript. All authors have read and approved the final manuscript. YC, HS, XZ and HB confirm the authenticity of all the raw data.

#### Ethics approval and consent to participate

All human procedures were performed in accordance with the Guidelines of the Human Research Ethics Committee of the Shandong Second Provincial General Hospital Affiliated to Shandong University (Jinan, China). The Human Research Ethics Committee of the Shandong Second Provincial General Hospital Affiliated to Shandong University (Jinan, China) approved the experimental protocols (approval no. XYK20181224). All participants agreed to use their samples for scientific research, and informed consent was obtained. All animal procedures were performed in accordance with the Guidelines of the Animal Care Committee of the Shandong Second Provincial General Hospital Affiliated to Shandong University (Jinan, China). The Animal Care Committee of Shandong Second Provincial General Hospital Affiliated to Shandong University approved the experimental protocols (approval no. XYK20181225).

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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