Ginkgo Biloba L. Extract Reduces H$_2$O$_2$-Induced Bone Marrow Mesenchymal Stem Cells Cytotoxicity by Regulating Mitogen-Activated Protein Kinase (MAPK) Signaling Pathways and Oxidative Stress

**Background:**
The oxidative stress environment of pathological tissue has an adverse effect on the survival of bone marrow mesenchymal stem cells (BMSCs) transplantation. Ginkgo biloba L. extract (EGB) has a potent antioxidant effect. In this research, we assessed the protective effects of EGB and EGB-Containing Serum (EGB CS) on BMSCs against injury induced by hydrogen peroxide (H$_2$O$_2$).

**Material/Methods:**
BMSCs were pretreated with EGB or EGB CS and treated with H$_2$O$_2$. The cell counting kit-8 (CCK-8) method was utilized to detect cell viability. The DCFH-DA Fluorescent Kit method was used to detect intracellular ROS level. Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and (CAT) were determined. The Hoechst staining assay and qRT-PCR assay were utilized to evaluate the effect of EGB on cell apoptosis. Mitogen-activated protein kinases (MAPKs) signaling pathway were detected by western blot analysis.

**Results:**
Compared to the H$_2$O$_2$ group, the number of apoptotic cells in the EGB and EGB CS pretreated groups significantly decreased. The mRNA expression ratio of Bax/Bcl-2 was also decreased. EGB and EGB CS can reduce the production of ROS in BMSCs exposed to H$_2$O$_2$. SOD, GSH-Px and CAT activities were significantly higher compared with those with H$_2$O$_2$ group. Furthermore, EGB or EGB CS pretreatment decreased the protein levels of p-p38MAPK and p-JNK in BMSCs compared to the H$_2$O$_2$ group.

**Conclusions:**
Our findings suggested that EGB and EGB CS have protective effect on BMSCs against oxidative stress injury and increase the survival rate of BMSCs transplantation by regulating p38MAPK and JNK signaling.

**MeSH Keywords:**
Apoptosis • Ginkgo biloba • MAP Kinase Signaling System • Mesenchymal Stromal Cells • Oxidative Stress

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Background

Bone marrow mesenchymal stem cells (BMSCs) are pluripotent stem cells that present therapeutic potential in regenerative medicine owing to their ability to self-duplicate, self-renew, proliferate, and differentiate into multilineage differentiation into bone, cartilage, muscle, endothelial cells, and islet-like cells, homing to diseased tissue and repairing it [1]. Bone marrow mesenchymal stem cells (BMSCs) was investigated as a method of regenerating these tissues for OA therapy [2]. Transplantation of BMSCs was reported to be a safe and effective treatment for T2DM in a macaque and rat model. The transplanted BMSCs also improved numerous bodily and cellular functions and repaired damaged tissue [3]. However, oxidative stress plays a vital role in osteoarthritis, diabetes, and other diseases. The study found a trend towards a positive correlation between radiographic severity of osteoarthritis and insulin resistance, and the results indicate that osteoarthritis might be correlated with impaired glucose metabolism. The study shows the importance of oxidative stress and metabolic disturbances in the pathogenesis of osteoarthritis [4]. The features of regulation of free radical oxidation in the blood and the synovial fluid have been studied in patients with knee osteoarthritis [5]. Over 90% of diabetic patients are classified as type 2 diabetes cases (T2DM), and one of the primary factors underlying the etiology of T2DM is insulin resistance caused by oxidative stress [6]. The persistent oxidative stress and inflammation environment in the lesion area is adverse to the survival of BMSCs, and over 80% of the transplanted cells die within the first 24 h after transplantation [7,8]. Therefore, promoting the survival of the transplanted BMSCs is a critical step to improve the treatment of osteoarthritis and diabetes.

Ginkgo biloba L. extract (EGB) is a traditional herbal medicine that has been shown to remove free radicals and has been used in neurology to improve circulation and blood supply [9,10]. EGB has antioxidant properties, and can reduce the hydrogen peroxide level in cerebellar neurons and protect cultured cortical neurons from iron-induced injury [11]. Previous studies have emphasized that Ginkgo biloba extract reduced generation of high-glucose-induced endothelial reactive oxygen species by regulating Akt/eNOS and p38 MAP kinase pathways [12].

It has been reported that the MAPK signaling pathway plays a crucial role in cell survival and oxidative stress [13–15]. Our previous study found that EGB can protect BMSCs against oxidative stress and decrease cell death rates of BMSCs in vitro [16]. However, whether the MAPK signaling pathway is involved in this process remain unclear. In the present study, we used cultured BMSCs and hydrogen peroxide (H₂O₂)-induced BMSCs apoptosis, demonstrating the protective effect of EGB and determined that EGB mediated the protective effect by altering the MAPK signaling pathway.

Material and Methods

BMSC culture

This research was approved by the Ethics Committee of Jilin University 2nd hospital. The BMSCs were collected from the long bones of 5-day-old Wistar rats (Laboratory Animal Center of Jilin University, China). The rats were euthanized by exposure to CO₂ and submerged in 75% ethanol for 5 min at room temperature to disinfect them. The femurs and tibias were removed from the muscles and connective tissue, and subsequently flushed with DMEM/F12 medium using a syringe. Cell suspension was centrifuged for 10 min at 1000 rpm. Then, the supernatant was removed and the cells were resuspended in DMEM/F12 medium supplemented with 10% FBS (GIBCO, USA) and adhered on a 100-ml flask at 1×10⁶ cells/flask. The culture medium was half-refreshed every 3 days and the cells were passaged into 2 flasks when the confluence reached 70–80%.

BMSC surface marker detection

BMSCs at passage 3 were collected to analyze the expression of CD34, CD45, CD19, and CD29 by flow cytometry. About 5×10⁴ cells were collected and incubated with mAbs against CD34, CD45, CD19, and CD29 for 30 min at room temperature, and then analyzed by flow cytometry. Flow cytometry was used to detect the expression of CD34, CD45, CD19, and CD29 of BMSCs.

Preparation of EGB-containing serum

EGB was obtained from Taiwan Chi Sheng Chemical Corp. The rat medicated serum was prepared according to the published protocols [17–19]. All procedures were approved by Jilin University Experimentation Committee and were carried out in accordance with the National Institute of Health guidelines of China for the care and use of laboratory animals. Briefly, 100 Wistar rats, aged 6–8 weeks old and weighing 200±20 g, were divided into experimental and control groups. The animals were supplied by the Laboratory Animal Center of Jilin University, China. Rats had free access to food and water and were kept in a 12/12 h light/dark cycle. Rats received an intragastric dose of EGB (12 mg/kg, which corresponds to 10 times the adult clinical dose). The control group received intragastric injections of physiological saline. At 60 min after administration, abdominal aortic blood was collected after the final drug administration, followed by centrifugation at 3500 rpm for 15 min. The serum samples were heat-inactivated at 56°C for 30 min, filtered with a 0.22-µm Millipore membrane filter, and stored at −80°C until use.

Cell treatment

The BMSCs were treated with either H₂O₂ at a final concentration of 400 μmol/l, EGB (Chi Sheng Chemical Corporation,
DA was used to measure intracellular levels of ROS in Her's protocol. The peroxide-sensitive fluorescent probe DCFH-assay kit (Beyotime Biotech, China) following the manufacturer's instructions. The MDA concentration and the SOD, CAT, and GSH-Px activity were calculated.

**Preparation of BMSCs homogenates**

The collected BMSCs were washed in PBS and lysed in lysis buffer (20 mM Tris base, 10 mM EDTA, 1% NP40, 10% glycerol, and 140 mM NaCl, pH 7.5) containing protease inhibitor (1 mM PMSF) for 30 min. Then, the homogenized cells were centrifuged at 4°C and 14 000 rpm for 15 min and the supernatants were subjected to MDA, SOD, CAT, and GSH-Px analysis.

**Measurement of MDA, SOD, CAT, and GSH-Px**

The MDA, SOD, CAT, and GSH-Px levels were measured using assay kits purchased from Nanjing Jiancheng Biotechnology Company (China). The MDA concentration and the SOD, CAT, and GSH-Px activity were calculated.

**Measurement of oxidative stress**

The levels of intracellular ROS were determined using an ROS assay kit (Beyotime Biotech, China) following the manufacturer's protocol. The peroxide-sensitive fluorescent probe DCFH-DA was used to measure intracellular levels of ROS in H₂O₂-induced BMSCs. Cells were harvested and then washed twice with PBS and incubated with DCFH-DA (10 mmol/L) at 37°C for 40 min in the dark for final analysis by fluorescence microscopy.

**Hoechst 33258 staining assay**

A Hoechst 33258 cell apoptosis staining kit (Beyotime, Nanjing, China) was used to confirm morphological changes in the nuclei. The transected cells were seeded onto sterile glass coverslips placed in 6-well plates and incubated for 24 h. The cells were fixed, washed twice with PBS, and stained with Hoechst 33258 staining solution for 5 min at room temperature in the dark. The cells were then washed twice with PBS, examined, and immediately photographed under a fluorescence microscope with an excitation wavelength of 350 nm. Apoptotic cells were defined by the condensation of nuclear chromatin or fragmentation to the nuclear membrane.

**Quantitative real-time PCR assay**

Total RNA was extracted from each group using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. DNase I was used to eliminate genomic DNA, the contaminant. The concentrations of isolated RNA samples were determined using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using a M-MuLV First-Strand cDNA Synthesis Kit (Sangon Company, Shanghai, China) according to the manufacturer’s instructions. The total reaction volume was 20 μL, which included 1 μg of total RNA, 10 ng/μL of random primers, and RNase-free distilled water.

qRT-PCR was performed using 2X SG Fast qPCR Master Mix (Sangon Company, Shanghai, China). The qRT-PCR instrument was equipped with a Roche LightCycler 480 detection system (Roche Diagnostics, Germany) according to the manufacturer’s instructions. Gene primer sequences are as follows: Bax, sense 5'-AGACACCTGAGCTACCCTGGAG-3' and anti-sense 5'-GTGGAAGTTGGCCTACGAAACA-3'. Bcl-2, sense 5'-TAAAAGCGCATCTGCACAC-3' and anti-sense 5'-CGTCTTCAGAGACAGCCAGAGGAG-3'. GAPDH, sense 5'-GGCACAGTCAAGGCTGAGAATG-3' and anti-sense 5'-ATGGTGGTGAAAGCCAGG-3'. The PCR reaction of each sample was repeated with 3 holes. Then, the mean threshold cycle was calculated. The results of fluorescence quantitative analysis were calculated by 2−DDCt, using the relative quantification (RQ) value statistics.

**Western blot assay**

The collected BMSCs were washed with cold PBS 3 times and then were lysed with lysis buffer containing protease and phosphatase inhibitors at 4°C, then centrifuged at 13 000 rpm for 15 min at 4°C. The protein concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology, China) according to the manufacturer’s instructions. Equal amounts of protein (20 μg/well) were loaded and separated by 10% polyacrylamide gel containing SDS electrophoresis and transferred to polyvinylidene difluoride membranes. After being blocked with 5% BSA in Tris-buffered saline containing Tween-20 for 2 h, the following primary antibodies were used: rabbit anti-P38 MAPK (1: 1000, CST, USA), rabbit anti-Phospho-P38 MAPK(Thr180/Tyr182) (1: 1000, CST, USA), rabbit anti-JNK (1: 1000, CST, USA), rabbit anti-Phospho-JNK (1: 1000, CST, USA), rabbit anti-Erk1/2 (1: 1000, CST, USA), rabbit anti-Phospho-Erk1/2 (1: 1000, CST, USA), rabbit anti-GAPDH (1: 1000, CST, USA), and HRP-conjugated secondary antibody (1: 2000, CST, USA). Immunoreactivity signals were developed
using the chemiluminescence of ECL reagent (PIERCE Chemical Co, IL, USA). The protein bands were quantitatively analyzed using a QuantityOne image densitometer.

**Statistical analysis**

Results are expressed as the mean ±SD from an appropriate number of experiments. One-way ANOVA followed by Bonferroni correction was used to compare the data among 3 or more groups, and the t test was also used. All statistical analyses were performed using the SPSS 17.0 software package for Windows (SPSS Inc., Chicago, IL) and a value of p<0.05 was considered significant.

**Results**

**Cell surface marker detection**

Adherent cells expressed CD29, but not the hematopoietic cell phenotype CD34, CD34, or CD19 (Figure 1). This confirmed that the adherent cells that were isolated and cultured from bone marrow were MSCs.

**EGB or EGB CS increased BMSC viability upon \( \text{H}_2\text{O}_2 \) injury**

To determine whether EGB plays a role in protecting BMSCs from oxidative stress-induced cell death, dissociated BMSCs were pretreated with EGB or EGB CS for 12 h followed by \( \text{H}_2\text{O}_2 \) treatment for 12 h, and cell viability was determined using the CCK-8 assay (Figure 2). BMSCs exposed to \( \text{H}_2\text{O}_2 \) had significantly lower cell viability compared with BMSCs protected by EGB or EGB CS (Figure 2).

**EGB or EGB CS ameliorated \( \text{H}_2\text{O}_2 \)-induced apoptosis**

To further assess the protective effect of the EGB or EGB CS against apoptosis in \( \text{H}_2\text{O}_2 \)-induced BMSCs, we performed Hoechst 33258 staining assay (Figure 3). Results showed that BMSCs treated with \( \text{H}_2\text{O}_2 \) displayed the typical changes of apoptosis. Pretreatment with the EGB or EGB CS for 12 h led to a significant decrease in the number of cells with condensed and fragmented nuclei. These results demonstrate EGB or EGB CS can decrease the development of apoptosis in BMSCs exposed to \( \text{H}_2\text{O}_2 \).

**Effects of EGB or EGB CS on the mRNA levels of Bcl-2 and Bax in BMSCs**

The Bcl-2 and Bax gene products were amplified by qRT-PCR and analyzed. Figure 4 shows that treatment with \( \text{H}_2\text{O}_2 \) elevated the ratio of Bax to Bcl-2. However, pretreatment with the EGB or EGB CS reduced the ratio of Bax to Bcl-2. These results show that EGB or EGB CS decreased the apoptosis of BMSCs caused by \( \text{H}_2\text{O}_2 \).
Increased intracellular reactive oxygen species (ROS) levels in BMSCs exposed to H\textsubscript{2}O\textsubscript{2}

Intracellular ROS production plays important roles in proliferation and apoptosis of various cell types. It has been reported that ROS production regulates aging, senescence, and osteogenic differentiation in BMSCs [20–22]. To further evaluate the effects of the EGB or EGB CS against ROS production in H\textsubscript{2}O\textsubscript{2}-induced BMSCs, we performed DCFH-DA staining assay. As shown in Figure 5, we found that H\textsubscript{2}O\textsubscript{2} treatment induced ROS production in BMSCs. Pretreatment with the EGB or EGB CS for 12 h led to a significant decrease in the number of cells with ROS production. These results demonstrate EGB or EGB CS decreased the intracellular ROS generation in BMSCs exposed to H\textsubscript{2}O\textsubscript{2}.

EGB or EGB CS lowered oxidative stress in BMSCs exposed to H\textsubscript{2}O\textsubscript{2}.

BMSCs exposed to H\textsubscript{2}O\textsubscript{2} had significantly higher levels of MDA and lower activities of SOD, CAT, and GSH-Px compared with those pretreated with the EGB or EGB CS for 12 h (Figure 6).

Effect of EGB or EGB CS on MAPK signaling pathways in BMSC cells

To further study the protective mechanism of EGB or EGB CS against BMSC oxidative damage, we analyzed the expression of p38MAPK, p-p38MAPK, JNK, p-JNK, ERK1/2, and p-ERK1/2 proteins by Western blot (Figure 7). The results indicated that the expressions of p-p38MAPK and p-JNK protein were significantly increased after the addition of H\textsubscript{2}O\textsubscript{2}. EGB or EGB CS pretreatment decreased the protein levels of p-p38MAPK and p-JNK in BMSCs compared to the H\textsubscript{2}O\textsubscript{2} group. There was no significant difference in the protein expression of p38MAPK, JNK, ERK1/2, and p-ERK1/2. EGB or EGB CS-mediated protection of BMSCs from apoptosis induced by H\textsubscript{2}O\textsubscript{2} was abolished. These results indicated that EGB or EGB CS prevented BMSC apoptosis mediated by oxidative stress through p38MAPK and JNK signaling pathways.

Discussion

EGB is an ideal antioxidant that has the ability to remove free radicals. In recent years, BMSC transplantation has been considered a promising treatment for diabetes [23]. After transplantation, BMSCs can settle down in the lesion area, and
Figure 5. Effects of EGB or EGB CS on the generation of ROS in BMSCs exposed to H$_2$O$_2$.

Figure 6. Oxidative stress in BMSCs exposed to H$_2$O$_2$ was alleviated by EGB or EGB CS. (A) MDA level changes in BMSCs treated with EGB or EGB CS; (B) SOD activities changes in BMSCs treated with either EGB or EGB CS; (C) CAT activities changes in BMSCs treated with either EGB or EGB CS. (D) GSH-Px activities changes in BMSCs treated with EGB or EGB CS. All data are presented as mean value ±SD and n=3 in each group. a) p<0.05, significantly different from control; b) p<0.05, significantly different from EGB; c) p<0.05, significantly different from EGB CS; d) p<0.05, significantly different from H$_2$O$_2$; e) p<0.05, significantly different from EGB + H$_2$O$_2$, one-way ANOVA.
As one of the most important ROS, hydrogen peroxide (H$_2$O$_2$) had been extensively used to induce oxidative stress in vitro models [27,28]. Apoptosis is triggered by changes in oxidative stress. Oxidative stress and subsequent ROS generation are 2 critical factors involved in protecting BMSCs from H$_2$O$_2$-induced apoptosis, and the imbalance between ROS generation and the innate ability of cells to scavenge the reactive species leads to increased oxidative stress [29,30]. ROS damage to polyunsaturated fatty acids and lead to higher levels of MDA that a major end product from membrane lipid peroxidation. H$_2$O$_2$ causes cell oxidative damage, mainly related to it attacking the antioxidant system [31]. The present results show that ROS production, MDA, and the total apoptosis were significantly increased, while the activities of antioxidant enzymes were significantly inhibited in BMSCs treated with H$_2$O$_2$. We administered EGB or EGB CS before H$_2$O$_2$ treatment, thereby protecting BMSCs from H$_2$O$_2$-induced apoptosis. We found that the nuclei of H$_2$O$_2$-treated BMSCs were degraded or condensed. However, a marked reduction in the number of apoptotic cells was found when BMSCs were pretreated with EGB or EGB CS for 12 h. These results confirmed that EGB or EGB CS could promote BMSCs proliferation, mainly by reducing apoptosis. Our results are similar to those mentioned in previous studies, showing that H$_2$O$_2$ treatment significantly decreased the activities of SOD, CAT, and GSH-Px in BMSCs, while the activity of these antioxidant enzymes was significantly increased in H$_2$O$_2$-induced BMSCs after EGB or EGB CS treatment. We found that multi-targeted antioxidant activity of EGB might protect tissues from oxygen radical damage.

Apopoptosis is a highly regulated and intrinsic cell-suicide program in which the Bcl-2 family plays a crucial regulatory role. The Bcl-2 family is a category of cytoplasmic proteins; Bax and Bcl-2 are 2 main members of this family that are functionally opposed, which promote apoptosis in various systems, depending on the Bax/Bcl-2 ratio [32]. In the present study, we demonstrated that EGB or EGB CS reduced apoptosis of BMSC treated with H$_2$O$_2$. Furthermore, EGB or EGB CS increased Bcl-2 and decreased Bax mRNA expressions in BMSCs, and EGB contributed to the attenuation of H$_2$O$_2$-induced apoptosis through regulation of the Bax/Bcl-2 ratio.
Many signaling molecules and pathways, such as NF-κB, PI3K/Akt, and MAPK, participate in oxidative stress-induced cell apoptosis [12,33]. Members of the MAPK family, one of the most important downstream signaling pathways of ROS, are generally considered to function as modulators of cell survival, proliferation, and apoptosis. The MAPK signaling pathway consists of 3 parts – ERK1/2, JNK, and p38MAPK – and is recognized to be closely related to cell apoptosis. Activation of the MAPK signaling pathway leads to enhanced oxidative damage [34]. The ERK1/2 cascade is involved in cell differentiation and proliferation, the JNK cascade modulates cell differentiation, apoptosis, and inflammation, and the p38 cascade may be involved in apoptosis and modulating the reactions of cytokines [35,36]. EGB or EGB CS-mediated protection of BMSCs from apoptosis induced by $H_2O_2$ was abolished. However, whether the protective effect of EGB or EGB CS on BMSCs is related to the MAPK signaling pathway is still unknown. Therefore, we examined the effect of EGB or EGB CS on MAPK signaling in BMSC oxidative injury. We first pretreated BMSCs with EGB or EGB CS for 12 h and then treated them with $H_2O_2$ for 12 h. The results indicated that EGB or EGB CS can reverse the decreased level of p-p38MAPK and p-JNK caused by $H_2O_2$ damage, but the protein expression of p-ERK1/2 was not significantly different.

These outcomes demonstrate that EGB or EGB CS can reduce the apoptosis of BMSCs by activating the p38MAPK/JNK signaling pathway. These findings also suggest that EGB or EGB CS may be combined with BMSCs transplantation in treatment of osteoarthritis, diabetes, and other diseases to promote the survival of transplanted BMSCs owing to its anti-apoptotic and antioxidant properties. However, the exact mechanism of this protective effect is not entirely clear, and we will assess the role of other regulatory factors involved in the p38MAPK/JNK signaling pathway in a future study.

Conclusions

Our findings suggest that EGB and EGB CS have protective effects on BMSCs against oxidative stress injury and can be used to improve the oxidative stress environment of osteoarthritis or diabetes and promote the survival rate of transplanted BMSCs by regulating p38MAPK and JNK signaling.

Conflicts of interest

None.

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