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Rosmarinic Acid Protects Adipose Tissue-Derived Mesenchymal Stem Cells in Nutrient-Deficient Conditions.

Ahmad Ghorbani[1], Hamid Reza Sadeghnia[1], Amir Reza Afshari[2], and Azar Hosseini[1]

[1]Pharmacological Research Center of Medicinal Plants, Mashhad University of Medical Sciences, Mashhad 91779-44553, Iran
[2]Department of Physiology and Pharmacology, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd 94149-74877, Iran

ABSTRACT: One of the major challenges for stem cell therapy of ischemic organs is that the transplanted cells are confronted with nutrient deficiency and oxidative stress. Previous studies have indicated that pretreatment of stem cells with cytoprotective phytochemicals improves their therapeutic potential. This study was aimed to investigate whether rosmarinic acid can enhance survival of adipose tissue-derived stem cells (ASCs) in nutrient-deficient culture as an *in vitro* model of ischemia. The ASCs were isolated from subcutaneous adipose tissue of male adult Wistar rats and incubated for 24 h with rosmarinic acid in nutrient-deficient (glucose- and serum-deprived, GSD) culture medium. In a separate experiment, ASCs were pre-incubated for 4 h with rosmarinic acid and then exposed to GSD conditions for 24 h. The viability of ASCs was determined using thiazolyl blue tetrazolium bromide assays. The effect of rosmarinic acid on the cell cycle was evaluated using propidium iodide staining. GSD conditions significantly decreased the viability of ASCs and enhanced the generation of reactive oxygen species (ROS), lipid peroxidation, sub-G1 cell populations, and necrosis. Both pre-incubation and incubation of ASCs with 0.75～6 μM rosmarinic acid significantly increased cell viability in GSD conditions. Rosmarinic acid further decreased the level of ROS, lipid peroxidation, the percent of cells in sub-G1 stage, and necrosis in GSD conditions. These findings suggest that rosmarinic acid enhances survival of ASCs cultured in nutrient-deficient conditions through promoting antioxidant effects. Therefore, rosmarinic acid may help preserve ASCs survival after they are transplanted into ischemic organs.

Keywords: necrosis, oxidative stress, rosmarinic acid, stem cells

INTRODUCTION

Mesenchymal stem cells (MSCs) have great potential for cell-based therapy as they can be obtained from adult tissues, expanded and differentiated in culture, and transplanted back into tissues (Ferrin et al., 2017; Volkman and Offen, 2017; Shafei et al., 2017). Although considerable progress has been recently made in MSC therapy, a number of challenges remain to be addressed before the most effective treatment can be achieved. A major challenges is that after transplantation into damaged tissues, exogenous cells are enter a pro-apoptotic environment caused by factors such as inflammatory cytokines, reactive oxygen species (ROS), and serum and O_2 deprivation (Geng, 2003; Zhang et al., 2001; Potier et al., 2007). This results in the loss of native or exogenous stem cells in the target tissue, which reduces the effectiveness of cell therapy. For example, it has been reported that approximately 17% of neural stem cells transplanted into spinal cords are apoptotic at 1 day (Mothe et al., 2008). In another study, almost all MSCs (>99%) injected into the infarcted myocardium of adult mice were dead on the fourth day (Toma et al., 2002).

Recently, many strategies have been used to enhance the survival of MSCs in pro-apoptotic conditions. These strategies include optimizing culture conditions, transducing MSCs with survival-related genes, improving adhesion of transplanted cells, preconditioning with non-lethal hypoxia, and pretreatment with growth factors. (Shi and Li, 2008). A number of studies have indicated that pretreatment with cytoprotective phytochemicals decreases stem cell apoptosis and improves their therapeutic potential (Ghorbani et al., 2018; Zhang et al., 2009; Li et al., 2014; Dadashpour et al., 2017). For example, ber-

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Correspondence to Azar Hosseini, Tel: +98-513-800-2283, E-mail: hoseiniaz@mums.ac.ir
Author information: Ahmad Ghorbani (Professor), Hamid Reza Sadeghnia (Professor), Amir Reza Afshari (Professor), Azar Hosseini (Professor)

berine, an isoquinoline plant alkaloid, has been shown to inhibit hypoxia-induced MSCs apoptosis by scavenging ROS and preventing liberation of pro-apoptotic signaling molecules from mitochondria (Zhang et al., 2009). Similarly, lycopene, a carotenoid phytochemical mainly found in colored vegetables, decreases ischemia-induced MSCs apoptosis through reducing the generation of ROS and subsequent oxidative damage (Li et al., 2014).

Rosmarinic acid, a polyphenolic phytochemical derived from several plant species, is a well natural antioxidants that displays cytoprotective effects in different models of oxidative stress (Ghaffari et al., 2014; Gao et al., 2005), inflammation (Han et al., 2017; Rahbardar et al., 2018), and chemical-induced toxicity (Jin et al., 2013). To our knowledge, there have been no previous reports investigating the effect of rosmarinic acid on viability of adipose tissue-derived MSCs (ASCs) in proapoptotic conditions. Therefore, we investigated whether rosmarinic acid can enhance survival of ASCs in glucose- and serum-deprived (GSD) culture as an *in vitro* model of ischemia.

MATERIALS AND METHODS

Animals

Six adult male Wistar rats (200～250 g) were used for isolation of ASCs. The animals were kept in a light- and temperature-controlled conditions (12-h dark/light cycle; 22±4°C) and had free access to normal laboratory chow and tap water *ad libitum*. Before beginning the study, all animal procedures were approved by the Animal Ethical Committee of Mashhad University of Medical Sciences (IR.MUMS.REC.1395.39).

ASCs isolation and characterization

The animals were anesthetized by intraperitoneal injection of 60 mg/kg ketamine (Rotexmedica GmbH Arzneimittelwerk, Trittau, Schleswig-Holstein, Germany) and 6 mg/kg xylazine (Rotexmedica GmbH Arzneimittelwerk). A sample of subcutaneous adipose tissue was removed from the left inguinal region and sliced into small pieces. The pieces of tissue were washed with phosphate-buffered saline (PBS) and incubated in Dulbecco's modified Eagle's medium (DMEM) containing 2 mg/mL of collagenase for 90 min at 37°C, while being shaken. After centrifugation (5 min, 500 *g*), the precipitated stroma-vascular fraction was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad CA, USA). After three passages, expression of MSCs-associated surface markers was assessed by flow cytometry using antibodies against CD29 (BD Biosciences, San Jose, CA, USA), CD34 (Novus Biologicals, Oakville, ON, Canada), CD45 (BD Biosciences), and CD90 (Novus Biologicals). In addition, the multipotency potential of isolated ASCs was examined by evaluating their ability to differentiate into adipocytes and osteoblasts, as described previously (Feizpour et al., 2014).

Incubation and pre-incubation with rosmarinic acid

The ASCs were seeded into 96-well plates containing normal culture medium (high-glucose DMEM supplemented with 10% FBS) and maintained overnight in a cell culture incubator. Cells were then treated for 24 h with 0.75～400 μM rosmarinic acid (Sigma, St. Louis, MO, USA) in normal medium to determine non-toxic concentrations of the test compound.

To evaluate the cytoprotective effect of rosmarinic acid in GSD conditions, normal culture medium was replaced with glucose- and serum-free DMEM containing 0.2～6 μM of the test compound. The cells were maintained in the GSD medium for 24 h. In a separate experiment, the ASCs were pretreated for 4 h with 0.2～6 μM of rosmarinic acid (in the normal medium) and then exposed to GSD conditions for 24 h.

Cell viability assays

The viability of ASCs cultured in 96-well plates was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) (Sigma), which is reduced to blue formazan crystals by living cells. The MTT reagent was prepared at a concentration of 5 mg/mL in PBS and 20 μL of the reagent was added to 200 μL of the cell culture medium (Hadjzadeh et al., 2006). After 4 h, the medium was discarded and 200 μL of dimethyl sulfoxide was added to each well. The optical density of each well was then read at 570 nm.

Measurement of intracellular ROS

The level of intracellular ROS was determined using dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Sigma), which enters cells where it is hydrolyzed by esterases to dichlorodihydrofluorescein. In the presence of ROS, H_2DCF-DA is oxidized to highly fluorescent dichlorofluorescein. After incubation of ASCs with rosmarinic acid in GSD medium, cells were washed with PBS and treated for 30 min with H_2DCF-DA (5 μM) at 37°C in the dark. The fluorescence intensity was measured at excitation/emission wavelength of 485/530 nm in a FLUOstar galaxy fluorescence plate reader (BioTek Instruments Inc., Winooski, VT, USA).

Lipid peroxidation assays

Levels of lipid peroxidation were estimated by measuring malondialdehyde (MDA), an end product of lipid peroxidation. After incubation of ASCs with rosmarinic acid in GSD medium, cells were scraped into trichloroacetic acid (2.5%, 1 mL) and centrifuged (2 min, 13,000 *g*) at 4°C. The lysate supernatant (500 μL) was added to a test tube

containing trichloroacetic acid (15%, 400 μL) and thiobarbituric acid (0.67%, 800 μL). The tube was vortexed, incubated in boiling water for 20 min, then cooled to stop the reaction and centrifuged (10 min, 1,000 *g*) at 4°C (Asadpour et al., 2014). The fluorescence intensity of the supernatant was measured at excitation/emission of 530 /550 nm.

Cell cycle assays

The ASCs cultured in 12-well plates were treated for 24 h with rosmarinic acid in GSD medium. Cells were then incubated for 30 min with propidium iodide (PI) (Sigma) reagent. The reagent consisted of 5 mg of PI (0.005%), sodium citrate (0.1%), and Triton X-100 (0.1% v/v), which permeabilizes the cell membranes and stains nucleic acids. The fluorescence intensities of PI-stained cells were determined using a FACSCalibur flow cytometer (BD Biosciences).

Annexin V-fluorescein isothiocyanate (FITC)/PI double staining assays

Annexin V/PI staining is used to detect early/late apoptotic cells. The ASCs were cultured in 12-well culture plates then incubated with rosmarinic acid (1.5～6 μM) in GSD conditions. After 24 h, cells were harvested, washed twice with ice-cold PBS and re-suspended in 500 μL of binding buffer. Next, 1 μL of FITC-conjugated annexin V (Abcam, Cambridge, UK) and 10 μL of PI were added to the buffer. After 10 min, the number of viable, early apoptotic, late apoptotic, and necrotic cells were determined using a BD Facscalibur™ flow cytometer (BD Biosciences). Data was analyzed by software FlowJo® vX.0.7 (Tree Star Inc., Ashland, OR, USA).

Statistics

Data were analyzed by one-way analysis of variance (ANOVA) and Dunnett multiple comparison *post hoc* test. Results were presented as mean±standard error of the mean (SEM) and $P<0.05$ was considered statistically significant.

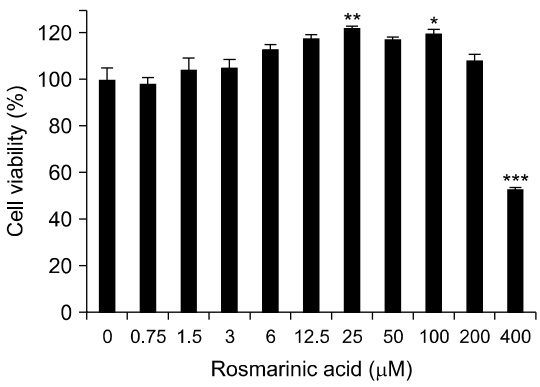

Fig. 1. Effect of rosmarinic acid on adipose tissue-derived stem cells (ASCs) cultured in standard medium. Cells were incubated with different concentrations of rosmarinic acid for 24 h. Cell viability was determined by MTT assays. Data are mean±SEM (n=6). $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared to untreated cells.

RESULTS

The effect of rosmarinic acid on cell viability in normal media

As shown in Fig. 1, rosmarinic acid at concentrations of 0.75～200 μM was not cytotoxic to ASCs, whereas concentration of 400 μM significantly reduced cell viability ($P<0.001$ vs. control).

Protective effect of rosmarinic acid in GSD conditions

Incubation of ASCs in GSD medium significantly decreased cell viability compared with cells maintained in standard medium ($P<0.001$). Co-incubation with rosmarinic acid (0.75～6 μM) significantly attenuated the cytotoxicity of GSD ($P<0.05$, Fig. 2A). In a separate experiment, ASCs were pre-incubated for 4 h with rosmarinic acid before they were exposed to GSD conditions for 24

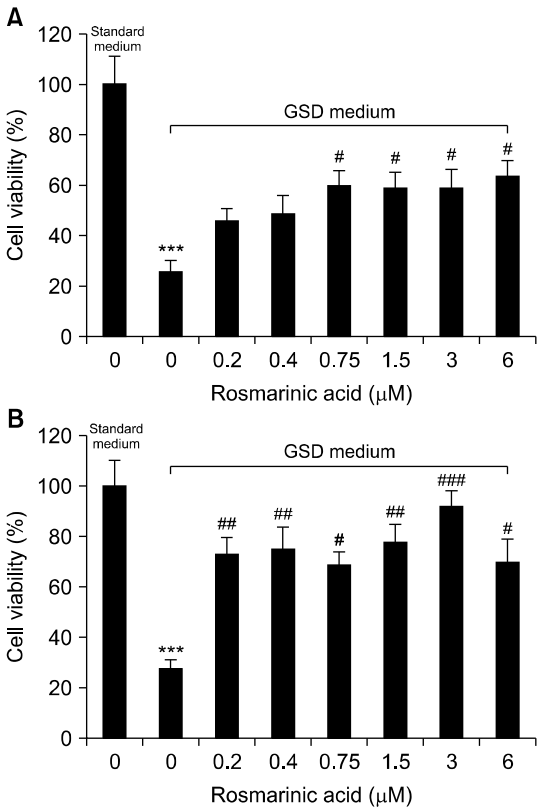

Fig. 2. Effect of rosmarinic acid on the viability of adipose tissue-derived stem cells (ASCs) cultured in glucose- and serum-deprived (GSD) conditions. (A) Cells were incubated for 24 h with rosmarinic acid in GSD conditions. B: cells were pre-treated with rosmarinic acid in standard medium for 4 h, and then cells exposed to rosmarinic acid in GSD conditions for 24 h. Data are mean±SEM (n=6). $^{***}P<0.001$ compared with untreated cells in standard medium. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ compared with untreated cells in GSD conditions.

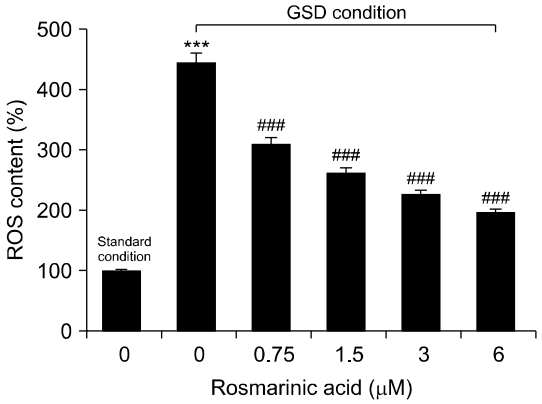

Fig. 3. Effect of rosmarinic acid on the level of reactive oxygen species (ROS) in adipose tissue-derived stem cells (ASCs) cultured in glucose- and serum-deprived (GSD) conditions. Data are mean±SEM (n=6). $^{***}P<0.001$ compared with untreated cells in standard medium. $^{###}P<0.001$ compared with untreated cells in GSD conditions.

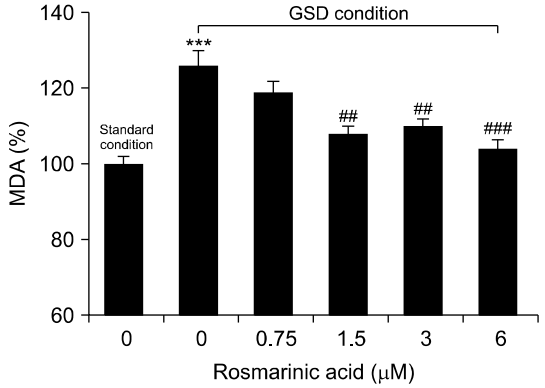

Fig. 4. Effect of rosmarinic acid on malondialdehyde (MDA) level as lipid peroxidation index in adipose tissue-derived stem cells (ASCs) cultured in glucose- and serum-deprived (GSD) conditions. Data are mean±SEM (n=6). $^{***}P<0.001$ compared with untreated cells in standard medium. $^{##}P<0.01$ and $^{###}P<0.001$ compared with untreated cells in GSD conditions.

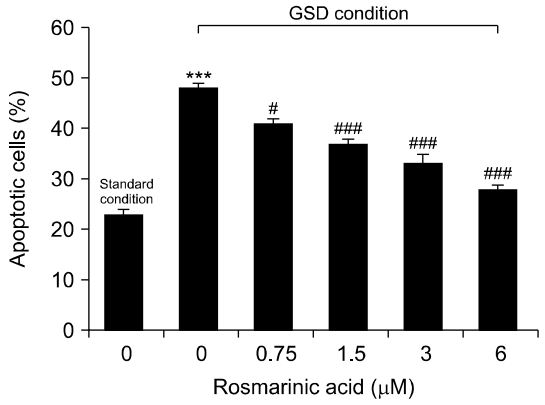

Fig. 5. Effect of rosmarinic acid on the percent of adipose tissue-derived stem cells (ASCs) in sub-G1 stage in glucose- and serum-deprived (GSD) conditions. Data are mean±SEM (n=3). $^{***}P<0.001$ compared with untreated cells in standard medium. $^{#}P<0.05$ and $^{###}P<0.001$ compared with untreated cells in GSD conditions.

h. Pre-incubation with rosmarinic acid (0.2~6 μM) significantly reduced GSD-induced cellular death ($P<0.05$, Fig. 2B).

Incubation with rosmarinic acid reduced GSD-induced oxidative stress

The generation of ROS in ASCs cultured in GSD condition was significantly higher than in cells cultured in standard medium (443±16% vs. 100±3%, $P<0.001$). Co-incubation with rosmarinic acid (0.75~6 μM) significantly decreased GSD-induced ROS generation in a concentration-dependent manner ($P<0.001$, Fig. 3).

Incubation with rosmarinic acid attenuated lipid peroxidation

Excessive ROS leads to subsequent lipid peroxidation and elevation of MDA. Incubation of ASCs in GSD conditions significantly enhanced the levels of MDA (126±4%, $P<0.001$) compared with cells cultured in standard medium (100±2%, Fig. 4). Co-incubation with rosmarinic acid (1.5~6 μM) significantly decreased the level of MDA in ASCs ($P<0.01$).

Incubation with rosmarinic acid decreased sub-G1 cell population

As shown in Fig. 5, culture of ASCs in GSD conditions increased the percent of cells with decreased DNA content in the sub-G1 stage compared with cells cultured in standard medium ($P<0.001$). Co-incubation with rosmarinic acid (0.75~6 μM) significantly decreased the percent of sub-G1 cells in a concentration-dependent manner.

Rosmarinic acid decreased GSD-induced necrosis in ASCs cells

As shown in Fig. 6A, GSD conditions increased cell death via inducing necrosis. Co-incubation with rosmarinic acid reduced necrosis and significantly increased the number of viable cells (Fig. 6B).

DISCUSSION

Stem cell therapy is considered an attractive option for treatment of many diseases (Larijani et al., 2012). Among, MSCs is more commonly used than other types of stem cells due to potential differentiation into cartilage, bone, ligament, muscle, tendon, adipose, and endothelial cells (Chamberlain et al., 2007). MSCs can also be easily harvested from different tissues such as bone marrow, adipose tissue, and skeletal muscle (Chamberlain et al., 2007). However, some pathophysiological conditions such as oxidative stress, inflammation, and tissue hypoxia lead to stem cell injury after transplantation into damaged tissues (Amiri et al., 2015). Increased level of

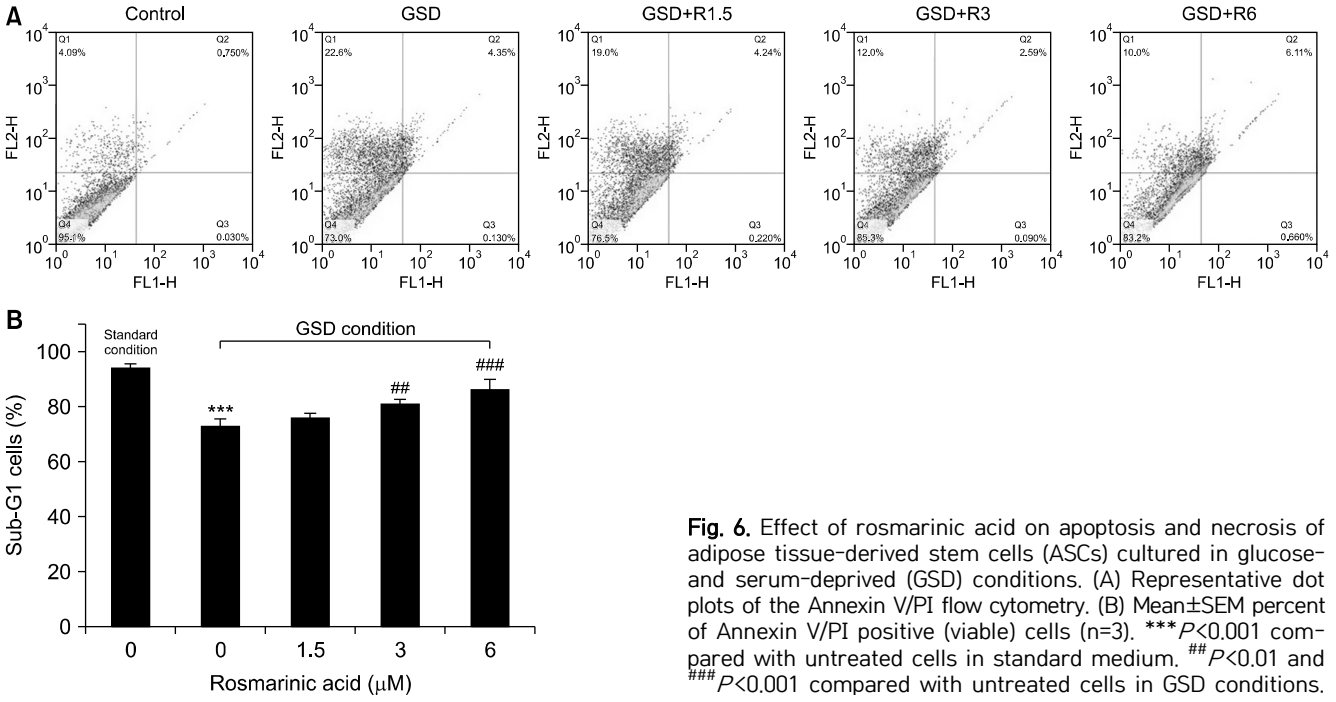

Fig. 6. Effect of rosmarinic acid on apoptosis and necrosis of adipose tissue-derived stem cells (ASCs) cultured in glucose- and serum-deprived (GSD) conditions. (A) Representative dot plots of the Annexin V/PI flow cytometry. (B) Mean±SEM percent of Annexin V/PI positive (viable) cells (n=3). $^{***}P<0.001$ compared with untreated cells in standard medium. $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared with untreated cells in GSD conditions.

ROS impact cellular processes of MSCs such as migration, adhesion, and proliferation, and may induce cell death (Yagi et al., 2013).

Rosmarinic acid, which is found in Labiatae herbs such as *Rosmarinus officinalis* (rosemary), *Melissa officinalis* (lemon balm), *Perilla frutescens* (perilla), and *Ocimum basilicum* (sweet basil) (Xu et al., 2008), has been reported to have anti-inflammatory (Rahbardar et al., 2017; Osakabe et al., 2002) and antioxidant (Tavafi, 2013) effects (Lucarini et al., 2014). In the present work, we evaluated whether rosmarinic acid can enhance survival of ASCs in GSD conditions as an *in vitro* model of ischemia-induced oxidative stress. Induction of GSD led elevated intracellular ROS, which in turn promoted lipid peroxidation and apoptosis in ASCs (Redza-Dutordoir and Averill-Bates, 2016). Consistent with these findings, other studies on bone marrow stem cells have shown that GSD conditions enhance cell death through promoting apoptosis (He et al., 2016).

Further, *in vivo* studies have suggested that lack of sufficient nutrients in damaged tissues, such as infarcted myocardium, may be one a major cause of MSC apoptosis (Geng, 2003). Our results show that both pre-incubation and incubation with rosmarinic acid may attenuate GSD-induced cell death by reducing levels of ROS and lipid peroxidation.

Several studies support our findings that rosmarinic acid has antioxidant activity (Luan et al., 2013; Zhang et al., 2015). Rosmarinic acid has been shown to reduce oxidative stress and cytotoxicity induced by *tert*-butylhydroperoxide and GSD in human neuroblastoma cells (Fallarini et al., 2009). Further, Zhang et al. (2015) reported that rosmarinic acid increases the activities of superoxide dismutase, catalase, and glutathione peroxidase, and decreases lipid peroxidation in the liver and kidneys of aging mice (Mushtaq et al., 2014). The protective effect of rosmarinic acid against lipid peroxidation has also been reported by other *in situ* and *in vivo* studies (Mushtaq et al., 2014).

Consistent with our results, a number of other studies have suggested that herbal compounds may attenuate oxidative stress in MSCs and therefore may be considered in clinical applications for maintaining viability and potency of MSCs (Ghorbani et al., 2018; Zhang et al., 2009; Yagi et al., 2013; Hsuuw et al., 2005; Li et al., 2016). For example, the naturally occurring polyphenols epigallocatechin-3-gallate and curcumin have been shown to attenuate H_2O_2-induced ROS accumulation and apoptosis in human MSCs and to restore their colony forming activity (Yagi et al., 2013). Further, Li et al. (2016) reported that dihydromyricetin, a natural polyphenolic compound that shows antioxidant activity, is protective against hydroxyl-induced DNA damage and apoptosis. These protective effects suggest a potential role for polyphenolic phytochemicals in MSC transplantation therapy, which should be validated in future *in vivo* studies.

In conclusion, the results of the present work suggest that rosmarinic acid enhances survival of ASCs cultured in nutrient-deficient conditions through its antioxidant activity. These findings may help preserve ASC survival after they are transplanted into ischemic organs.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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