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Mesenchymal stem cells proliferation augmentation by preconditioning with resveratrol (*In vitro* Study)

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ABSTRACT

Amelioration of culture conditions of Mesenchymal Stem Cells (MSCs) and optimization of preconditioned micro-environments can increase the numbers of cultured and expanded of MSCs in vitro then used for therapeutic applications. Cell proliferation and survival rates are the main hurdles in the clinical use of MSCs. Cultivated MSCs Survival and proliferation are impaired by oxidative stress and pre-apoptotic factors released during cultivation processes. Hence, strategies that reduce cell death and elevates the viability and proliferation of MSCs will be added values for using MSCs in therapeutic uses. The present study discussed the efficiency of resveratrol (RSV) to ameliorate the survival and proliferative capacity of MSCs when used in the preconditioned medium of MSCs preparation procedures. It was revealed that MSCs pretreated with 10 µM of RSV in vitro exhibited a significant proliferation than cells not treated with RSV. RSV suppressed the apoptotic condition, stress of oxidation and cell death probabilities through its potential effect on cytoprotective gene Bcl-2 in addition regulating Bcl-2/Bax ratio.

1. INTRODUCTION

Mesenchymal stem cells have been widely studied as therapeutically trials of regeneration for various injuries and diseases. Particularly, MSC preparation techniques have been expanded as a promising strategy to enhance the regenerative potential of MSC. Moreover, the application of spontaneous stem cells has modified great interest because of their characteristic of transplantation and reduction of autoimmunity problems. Transplantation of MSCs to the same individual isolated from has been extendedly evaluated in many therapeutic studies for several diseases, with promising results and dependent competencies (Wang et al., 2018a).

Resveratrol (RSV), a polyphenolic non-flavonoid plant with a chemical stilbene group, was discovered in the extract of many plants as white hellebore and Polygonum cupsidatum in 1963 (Safaeinejad et al., 2018). Resveratrol is extendedly used as an alternative medicinal plant. RSV has many effective actions as inhibition of oxidative molecules as reactive oxygen and nitrogen species and eliminate the excessive oxidative free radicals mainly through the beneficial action of its hydroxyl groups (4-OH, 3-OH, and 5-OH). Furthermore, the natural plant resveratrol has a vast therapeutic capacity according to its protective effects against oxidation, inflammation, and cancer conditions for diverse tissues against acute or chronic diseases. RSV also, has a beneficial effect in improving MSCs properties and its therapeutic role by increasing their chances of existence, self-renewal, lineage fidelity, and anti-aging impact (Hu and Li, 2019).

Inflammation and microglia activity are reduced in all processes described above, resulting in a decrease in inflammatory edema. Finally, RSV supplementation causes a decline in p53 expression and enhancement of protective pathway by increasing the expression of B-cell lymphoma 2(Bcl-2) in response to pro-apoptotic pathology Bcl-2-associated X protein (Bax), as well as any of the above-mentioned pathways (Zhang et al., 2017). As an outcome, a decrease in the expression of caspase 3, causes an increased cell survival. It was reported that RSV treatment raises the amount of effective MSC engraftments in AD-deficient mice's brains. Furthermore, other studies have proved that RSV possesses molecular effects on the cognitive and behavioral functions of AD model of rats leading to those rats treated with RSV to show a normality in Morris Water Maze test and an improvement of the outcomes of radial arm maze and open field test. Most of later preclinical studies revealed that RSV has a beneficial effect on decreasing Aβ, Neurofibrillary tangles (NFTs), and Advanced glycation end products (AGEs) synthesis then inhibits the main pathogenic pathways of AD in addition affecting oxidative stress, and inflammation. Pharmacodynamically, RSV enhances Sirtuin 1 (SIRT1), reduces the stresses of oxidation and promotes normal cleavage of Amyloid-beta precursor protein (APP) in the non-amylogenic route keeping non-pathogenic abnormal pathway away (Wang et al., 2018b).

In culture of neuronal cells, it was discovered that when Aβ is present, Bax (a pro-apoptotic protein) expression elevates with time, while Bcl-2 (an anti-apoptotic protein) expression declines. In the hippocampus, non-soluble amyloid β fragments increases the levels of Bcl-2 interacting mediator of cell death decreasing Bcl-2 level, hence, results in Bax activation, and subsequent accelerated programmed cell death process. Bax inhibition can prevent,

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Aβ-induced death (Kudo et al., 2012). In a rat model of Alzheimer's disease, overexpression of Bcl-2 inhibited caspase-9 activation and reduced the cleavage of tau protein induced by caspases. Moreover, over expression of Bcl-2 decreased the processing of abnormal excessive cleavage of Amyloid-beta precursor protein (APP) and tau, reduced the quantities of Neurofibrillary tangles (NFTs) and extracellular particles of Aβ, and decrease the neurotoxicity which enhanced the position of awareness (Rohn et al., 2008).

Apoptosis is a form of programmed cell death that helps maintain fetal and adult tissue homeostasis. The existence of functional apoptotic pathways in fetal MSCs has been shown. MSCs encoding an anti-apoptotic Bcl-2 gene showed increased cell viability, confirming a recent theory. (Oliver et al., 2011).

Alzheimer's Disease International, mentioned that 46.8 million people in 2015 affected with dementia worldwide and 60-80% of this huge number was suffering from that Alzheimer's disease (Alzheimer Association, 2018). Moreover, it is predicted that, these numbers will jump to double after each 20 years. By 2030 it will reach74.7 million and 131.5 million in 2050 (Prince et al., 2016). Dementia is likely to be one the most difficult diseases to treat in developed countries, with significant social and economic consequences. In 2015, the global costs of dementia were reported to be $818 billion US dollars. As a result, as soon as possible, successful care should be investigated and enforced (Wimo et al., 2017).

Kálai et al. (2011), revealed that dementia's complicated function necessitates at least a few different clinical methods. The current emphasis is on reducing the adverse consequences of amyloid and neurofibrillary tangle aggregation, as well as inhibiting amyloid formation. Nevado-Holgado and Lovestone (2017) mentioned that anti-inflammatory medications are also given to patients with advanced Alzheimer's disease as part of their clinical care. Patients treated with non-steroidal anti-inflammatory drugs (NSAIDs), such as in the case of coronary disease, dementia and Alzheimer's disease are less common among them, according to scientific evidence.

Brain-derived neurotrophic factor BDNF promotes noradrenergic and serotonergic neuron production and protects them from toxicity. With its positive effect on dendrite formation, it regulates neuronal continuity and plasticity. In cells and tissues, oxidative stress is described as a rise in the release of reactive oxygen species (ROS) and/or a depletion of the antioxidant protection system, resulting in an imbalance between prooxidants and antioxidants, which can lead to harm. ROS will target polyunsaturated fatty acids in the bio membrane, causing free radical chain reactions and lipid peroxidation to increase (Omayma et al., 2017a). Oxidative stress has been linked to several diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), atherosclerosis, cancer, and diabetes. AC reduced glutathione (GSH) content and anti-ROS activity in the brain (Omayma et al., 2017b).

The present study aimed to investigate the function of RSV in enhancing the proliferation of MSCs in rats which may be a potential therapeutic technique for neurodegenerative diseases.

2. MATERIAL AND METHODS

2.1. Stem Cells isolation:

The tibiae and femurs of 6-week-old male rats would be flushed with Dulbecco's adapted Eagle's medium (GIBCO/BRL) mixed with 10% fetal bovine serum (GIBCO/BRL) to extract bone marrow. A density gradient was used to distinguish nucleated cells [Ficoll/Paque (Pharmacia)] and resuspended in full culture medium containing 1% penicillin streptomycin (GIBCO/BRL) and 10 M RSV. As a primary culture, cells were incubated for 12-14 days at 37°C in 5% humidified CO_2. Per 2-3 days, the media was replaced. As wide colonies formed (80-90 percent confluence), the cultures were washed twice with phosphate buffer saline (PBS) before being trypsinized for 5 minutes at 37°C with 0.25 percent trypsin in 1 mM EDTA (GIBCO/BRL). Cells were resuspended in serum-supplemented medium and incubated in 50 cm2 culture flasks after centrifugation (Falcon). The societies that resulted became known as first passage cultures. The adherent colonies of cells is trypsinized and recorded on day 14.MSCs isolated from bone marrow were selected and cultured in accordance with previously published protocols (Choi et al., 2019)**.**

2.2. Proliferation

96-well plates were seeded with BMMSCs (11000 cells/well). Following the normal procedure, cell proliferation was measured using a cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan). In summary, 10 µL of CCK-8 solution for each 100 µL of medium was injected into wells that were generated on a daily basis. After being mixed, the plates were incubated for 2 hours in a 37 °C, saturation humidity, and 5% CO2 atmosphere. A microplate reader (Bio-TEK Instruments, Winooski, VT, USA) was used to measure the OD value at 450 nm. From Day 0 to Day 6, assays were carried out (Shuai et al., 2016).

2.3. Gene expression by qRT-PCR for mRNA of BCL-2 and Bax

Lymphocytes were collected 4 hrs following to irradiation for ribonucleic acid (RNA) isolation from the cultures. The cells were washed with PBS, and then RNA was extracted by the TriPure reagent (Roche Applied Science, Germany) according to the manufacturer's recommendations. RNA sediments were dissolved in 20 µl Diethyl pyrocarbonate (DEPC)-treated RNase-free water and were assessed by electrophoresis on agarose gel. First-strand cDNA was synthesized from 1 µg of total RNA with oligo (dT) 18 primer using the Revert Aid ™ First Strand cDNA Synthesis kit (Fermentas, Germany) in the same way as was described in our previous study. Gene expression assessments were performed on a StepOne (48-well) real-time PCR system (Applied Biosystems) by SYBR® Premix Ex Taq™ (Takara, Japan) as described previously (Hosein et al., 2018). Relative quantitative real-time PCR method was employed to assess *Bax* and *Bcl-2* gene expression levels (table 1).

Table.1 Rats primers sequences of BCL-2 and Bax for qRT-PCR (Zheng et al., 2018)**:**

Gene	Sequence (5'-3')
Bcl-2	Forward: TACTTAAAAAATACAACATCACAG
	Reverse: GGAACACTTGATTCTGGTG
Bax	Forward: GCTTCAGGGTTTCATCCAG
	Reverse: GGCGGCAATCATCCTCTG
B-actin	Forward: GGAGATTACTGCCCTGGCTCCTA
	Reverse GACTCATCGTACTCCTGCTTGCTG

2.4. Statistical analysis

To determine the differences between two groups, GraphPad prism software was used (version 6.0; GraphPad, La Jolla, CA, USA). The data are presented as the mean ± standard error. $P< 0.05$ was set for statistical significance.

3. RESULTS

The results (Table 2& Figures 1-3) showed a significant increase in proliferation and mRNA gene expression of Bcl-2 in preconditioned MSC with resveratrol than non-preconditioned MSC

Table.2 Effect of RSV in preconditioned medium of MSC on MSCs proliferation, expression of Bax and Bcl-2.

Groups parameters	MSC group	MSC+ RSV group
Proliferation	98.58 ± 0.43	127.5 ± 6.9[a]
mRNA of Bax	1.0 ± 0.004	0.06 ± 0.02[a]
mRNA of Bcl-2	1.02 ± 0.015	6.46 ± 0.58[a]

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same column are significantly different at (P<0.05). [a] significant at P≤0.05 compared to MSCs group.

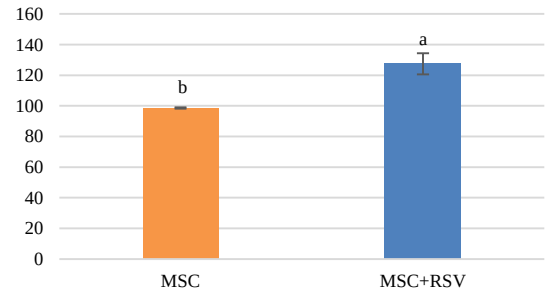

Fig. 1 MSC proliferation with and without pretreating with 10 µM of RSV. The proliferation results were expressed as mean ±SE. [a,b] = significant at P≤0.05

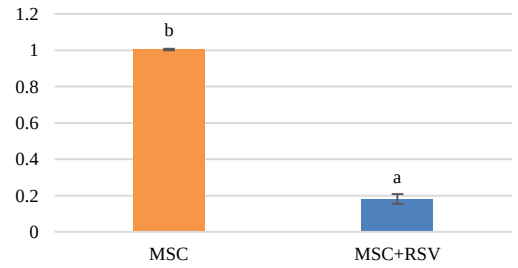

Fig.2. qRT-PCR for expression of Bax mRNA with and without pretreating with 10 µM of RSV. The results were expressed as mean ±SE. [a,b] = significant at P≤ 0.05

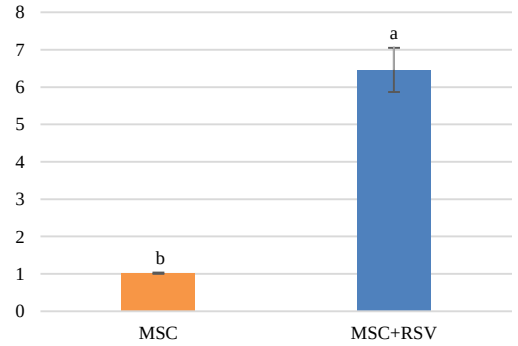

Fig.3. qRT-PCR for expression of Bcl-2 mRNA with and without pretreating with 10 µM of RSV. Values were expressed as mean ±SE. [a,b] = significant at P≤ 0.05.

4. DISCUSSION

MSCs are used for tissue engineering, but during in vitro processing, which is needed to produce enough number of cells for disease treatment, they usually lose their stem cell characteristics. It is important for the advancement of stem cell therapy for MSCs to retain their stem cell characteristics during long-term in vitro expansion (Choi et al., 2019). We examine the function of RSV in MSC preconditioning medium in improving MSC proliferation and differentiation in culture, which can be used for stem cell therapy and has adequate efficacy and protection for clinical usage.

Apoptosis reduction or resistance to apoptotic pathways is essential for MSC proliferation. In patients with neurodegenerative disorders, postmortem experiments showed enhanced apoptosis in the hippocampal subfields and entorhinal cortex (Lucassen et al., 2001). The function of resveratrol in the preconditioning medium of MSC was investigated in this research. Chronic stress and neurotoxicity have been shown to decrease Bcl-2 immunological reactivity and lower Bcl-2 mRNA expression is linked to higher Bax mRNA expression (Luo et al., 2004).

In support of this hypothesis, we emphasized that RSV increases proliferation, increases Bcl-2, and decreases Bax, confirming its role in decreasing apoptosis via its previously demonstrated role in activating SIRT1, which inhibits H_2O_2-induced cell death and overturns it by preventing the phosphorylation of ERK caused by ROS development, and resveratrol has a cytoprotective effect by attenuating ERK phosphorylation (Yoon et al., 2015).

Previous results had been done by Kosten et al (2008) revealed that the expression of Bcl-2 mRNA was raised in the hippocampus and PFC, and Bax mRNA expression was regulated in the hippocampus, with resveratrol therapy bringing both amounts to normal. In addition, it was found that frequent periods of unpredictable stress had little effect on Bcl-2 mRNA expression in the hippocampus of those treated with RSV. This previous work confirms our results indicating the beneficial effects of RSV on MSC proliferation through increasing Bcl-2 and decreasing of Bax expression.

Importantly, in humans, the BDNF is linked to reduced hippocampal volume and cognitive deficits (Chen et al., 2005). Furthermore, animal experiments have shown that decreased BDNF expression or release is adequate to replicate the neuronal atrophy induced by persistent stress. Up-regulation of BDNF expression or release, on the other hand, causes an antidepressant reaction, which is linked to improved new neuron survival (Banasr et al., 2008). Resveratrol has been shown to prevent cell death by inhibiting ROS formation, caspase-3, and Bax processes, as well as upregulating Bcl-2 function (Bournival et al., 2009).

Standard aerobic metabolism produces reactive oxygen species (ROS), which include oxygen ions, oxygen-free radicals, and peroxides. Multiple signaling mechanisms, including cell proliferation, survival, and inflammation, are regulated by ROS. ROS-related diseases such as infection, cell death, and neurodegeneration result from a mismatch between ROS levels and antioxidant activity. BCL-2 is a well-known apoptosis inhibitor. Cells with a higher ratio of BCL-2 to BCL-2-associated X protein (BAX) are less receptive to pathological stimuli and are unable to react to apoptotic signals (Russell et al., 2015).

The use of cytoprotective genes increases proliferation after preconditioning with ROS scavenging agents. MSCs with higher Bcl2 levels have better angiogenesis and survival rates, as well as lower apoptosis. MSC longevity and potentiality increase as culturing and microenvironment conditions improve. Hence the ability of RSV to regulate the Bcl2/Bax ratio by inhibiting apoptosis pathways and

scavenging ROS and Oxidative free radicals increases MSC proliferation (Wang et al. 2014).

5. CONCULOSION

From the present study, we could conclude the possible mechanism of using RSV during MSCs preparation process. 10 µM concentration of resveratrol could activate antioxidation condition by increasing cytoprotective gene and regulating the Bcl-2/Bax ratio which increased cell survival and proliferation.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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