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Carvacrol promotes angiogenic paracrine potential and endothelial differentiation of human mesenchymal stem cells at low concentrations

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ABSTRACT

Objectives: Phenolic monoterpene compound, named Carvacrol, has been found to exert different biological outcomes. It has been accepted that the angiogenic activity of human mesenchymal stem cells was crucial in the pursuit of appropriate regeneration. In the current experiment, we investigated the contribution of Carvacrol on the angiogenic behavior of primary human mesenchymal stem cells.

Methods: Mesenchymal stem cells were exposed to Carvacrol in a dose ranging from 25 to 200 μM for 48 h. We measured cell survival rate by MTT assay and migration rate by a scratch test. The oxidative status was monitored by measuring SOD, GPx activity. The endothelial differentiation was studied by evaluating the level of VE-cadherin and vWF by real-time PCR and ELISA analyses. The content of VEGF and tubulogenesis behavior was monitored *in vitro*. We also conducted Matrigel plug *in vivo* CAM assay to assess the angiogenic potential of conditioned media from human mesenchymal stem cells after exposure to Carvacrol.

Results: Carvacrol was able to increase mesenchymal stem cell survival and migration rate ($p < 0.05$). An increased activity of SOD was obtained while GPx activity unchanged or reduced. We confirmed the endothelial differentiation of stem cells by detecting vWF and VE-cadherin expression ($p < 0.05$). The VEGF expression was increased and mesenchymal stem cells conditioned media improved angiogenesis tube formation *in vitro* ($p < 0.05$). Moreover, histological analysis revealed an enhanced microvascular density at the site of Matrigel plug in CAM assay.

Conclusions: Our data shed lights on the possibility of a Carvacrol to induce angiogenesis in human mesenchymal stem cells by modulating cell differentiation and paracrine angiogenic response.

1. Introduction

Angiogenesis is a sophisticated multi-step process of new blood vessels formation with the coordinated participation of cells and different factors (Auerbach and Auerbach, 1994; Bergers et al., 2003). Endothelial cells (ECs) are the main component of this scenario involved actively in the development and maturation of nascent blood vessels (Bergers et al., 2003). Insufficient angiogenesis was found to limit tissue reconstitution and remodeling rate, leading to ischemic and degenerative changes (Herbert and Stainier, 2011). Previous works by our group and others revealed that human bone marrow mesenchymal stem cells (hMSCs) had the capability to differentiate into different cell types, peculiarly endothelial lineage and secrete pro-angiogenic factors (Aysa Rezabakhsh et al., 2016; Tao et al., 2016). With respect to unmatched features of hMSCs, novel approaches and methods for promoting hMSC differentiation into different mature cell types are under investigation to improve the quality and quantity of tissue repair (Udalamaththa et al., 2016). In recent cell therapy medicine, limitless or uncontrolled use of recombinant factors and differentiation agents may represent side effects while imposing extreme laboratory costs

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(Udalamaththa et al., 2016). Regarding these disadvantages, most of the research centers in the world are looking for examining alternative natural products in the field of regenerative medicine (Jiménez-Arellanes et al., 2016).

Carvacrol - the main monoterpene phenol – can be found as an herbal metabolite in the family Labiatae including Satureja, Thymbra, Origanum, Corydothymus and Thymus species (Kirimer et al., 1995). Through the decades, this metabolite was extensively used as a source of flavor in foodstuffs with regard to their possible antitumor activity (Aeschbach et al., 1994). For instance, human hepatocarcinoma and larynx tumor cells treated with Carvacrol represent the apoptotic phenotype (Stammati et al., 1999). Using the higher concentrations, Carvacrol promoted tumor atresia by the down-regulation of CDK4 and CCND1 while up-regulating CDK inhibitor P21 (Dai et al., 2016). Both DNA-protective ability and dose-dependent cytotoxic effect of Carvacrol have been documented in the scientific literature (Slamenova et al., 2006; Sobral et al., 2014). The protective effect of Carvacrol could correlate with the protection of antioxidant system and reduction of nitric oxide level in damaged cells (Jayakumar et al., 2012). It was found Carvacrol could diminish the iNOS level in bovine aortic ECs exposed to lipopolysaccharide (Guimarães et al., 2012). Different effects of Carvacrol on various cell types may be related to variations in the concentrations used. Suntres and co-workers noted various biological and pharmacological activities of Carvacrol. It was thought that Carvacrol at biological, pharmacological and toxicological concentrations yielded different outcomes (Suntres et al., 2015).

Given the importance of natural bioactive compounds in the realm of regenerative medicine and the inevitable importance of angiogenesis, we aimed in the current work to elucidate the potential angiogenic effect of Carvacrol on primary hMSCs. To determine the differentiation potency of hMSCs to endothelial phenotype and angiogenic paracrine response, hMSCs were incubated with a smaller concentration of Carvacrol for 48 h. The angiogenic potential of hMSCs after exposure to Carvacrol was examined *in vitro* and *in vivo*.

2. Material and methods

2.1. Ethical issue

All procedures performed through the current experiment, involving human participants, were in accordance with the Local Research Ethic Committee of Tabriz University of Medical Sciences and ethical principles of the Declaration of Helsinki.

2.2. hMSCs isolation and culture

Human bone marrow samples were collected from healthy volunteer donors after obtaining informed consent. Samples were collected in tubes containing 6000 IU of sodium heparin and mononuclear cells (MNCs) separated by using Ficoll density gradient, centrifuged at 4000*g* for 30 min at 4 °C. After twice washing with PBS, MNCs were suspended in low-glucose Dulbecco's Modified Eagle's medium (DMEM/LG; Gibco) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Biosera) and kept at 37 °C in a humidified atmosphere with 5% CO_2. Cells at passage 3 were subjected to different analyses.

2.3. Flow cytometric characterization of isolated hMSCs

Immunophenotypic profile of cellular subsets was performed by using the surface markers panel including PE mouse anti-CD31 (Cat No: 12-0319-4; ebioscience), FITC mouse anti-CD133 (Cat No: 130-105-226; Miltenyi Biotec), FITC mouse anti-CD34 (Cat No: 11-0441-81; BD), and PE mouse anti-VEGFR-2 (Cat No: 580494; BD) and FITC mouse anti-CD44 (ebioscience). Isotype control antibodies were also used to exclude background staining. 100 µl blocking buffer (ebioscience) containing an appropriate concentration of each antibody was added to cell pellets and maintained at 4 °C for 40 min. We performed the flow cytometric analysis by using an FACSCalibur Flow Cytometer (BD Biosciences) and FlowJo software ver. 7.6.1. Flow cytometric analysis was set in triplicates.

2.4. Determination of Carvacrol cytotoxicity on hMSCs

hMSCs at a seeding density of 1×10^4 were transferred to each well of 96-well plates. After 24 h, hMSCs were exposed to various concentration of Carvacrol in a dose ranging from 25 to 200 µM. Parallel control was used without the addition of Carvacrol. After 48 h, supernatants were removed and replaced with 20 µl 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; 5 mg/ml). Next, 100 µl of DMSO solution was added to each well. The absorbance was read by a Microplate Reader (BioTek) at 570 nm using a reference wavelength of 630 nm. Results were expressed relative (%) to the OD value of parallel time-matched controls. In our study, the final concentration of DMSO was below 0.05%. Each experiment was performed 3 times in octuplicate to confirm the consistency of data.

2.5. Oxidative status evaluation

The effect of Carvacrol on hMSCs oxidative status was assessed by the evaluation of superoxide dismutase (SOD) and Glutathione peroxidase (GPx) activity. hMSCs were exposed to Carvacrol concentrations, including 12.5, 25 and 50 µM; below the IC_{50} value for a period of 48 h. Cells were lysed using protein lysis buffer (150 mM NaCl, 0.1% SDS, 50 mM Tris-HCl, 2 mM EDTA, 1% NP-40) and centrifuged at 14000 rpm for 10 min at 4 °C. The activity of SOD was monitored by using 0.06% pyrogallol solution at 420 nm. GPx activity was measured in accordance with the previous protocols (ZB-GPX48, Zellbio GmbH). This assay was performed in triplicate.

2.6. In vitro scratch assay

hMSCs were plated in 24-well plates at initial seed densities of 5×10^5 cells per well in 500 µl culture medium. Upon reaching confluence, the hMSCs monolayer was scratched in a straight line with a sterile yellow pipette tip and exposed to different concentrations of Carvacrol, 12.5, 25 and 50 µM for 72 h. The migration properties were quantitatively measured by determining the average distance between two sides of a scratch region in randomly 7 serial microscopic high power fields. The migration rate was calculated as the ratio of the final migrated area to the initial denuded area expressed as µm by using ImageJ version 1.49.

2.7. Real-time PCR analysis of hMSCs differentiation into ECs

The expression of lineage specific genes such as VE-Cadherin and von Willebrand factor (vWF) were detected during differentiation of hMSC toward the EC-like cell. Transcription level of VEGF was studied to examine the potential paracrine angiogenic of hMSCs under treatment with Carvacrol. Total cellular RNA was extracted using RNA extraction kit (Yekta Tajhiz Co). cDNA was synthesized by Bioneer cDNA synthesis kit (Cat no: k-2046, Bioneer). Quantitative RT-PCR was performed using a Light Cycler instrument Rotor-Gene 6000 and the SYBR Green real-time PCR kit (ebioscience). The sequence of used primers was depicted in Table 1. The expression of GAPDH used as an internal housekeeping gene. Three sets of experiments were done for each gene.

2.8. Measuring protein level of VE-Cadherin by ELISA assay

ELISA assay was designed by our group. 100 µl of mouse anti-human VE-Cadherin (1 µg/ml; Cat No: MAB938; R & D) was transferred to each well of 96-well plates (polystyrene-treated plated, SPL) and kept overnight at 4 °C. After blocking 1% BSA solution, 50 µl of cell lysate