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Carnosol promotes endothelial differentiation under H_2O_2-induced oxidative stress

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Abstract: Oxidative stress causes deregulation of endothelial cell differentiation. Carnosol is a potent antioxidant and antiinflammatory compound. In the present study, we examined whether the antioxidant effect of carnosol might protect bone marrow stem cells against H_2O_2-induced oxidative stress and promote endothelial differentiation. We examined cell viability by the MTT assay; oxidative stress and apoptosis were analyzed through changes in ROS levels, apoptotic ratio and caspase-3 activity; changes in protein expression of OCT-4, Flk-1, CD31 and Nrf-2 were assessed by Western blot analysis. H_2O_2 treatment increased oxidative stress and reduced cell viability, while the stem cell marker OCT-4 and endothelial markers Flk-1, CD31 were significantly downregulated as a result of the treatment with H_2O_2. Treatment with carnosol improved the antioxidant status, increased OCT-4 expression and promoted endothelial differentiation. This study provides evidence that carnosol could increase the antioxidant defense mechanism and promote endothelial differentiation.

Key words: carnosol; differentiation; endothelial cells; Nrf-2; oxidative stress; ROS

INTRODUCTION

Circulating endothelial progenitor cells (EPCs) have been identified to be involved in early and postnatal neovascularization [1,2]. Circulating EPCs are biomarkers in cardiovascular diseases, which ultimately lead to atherosclerosis [3-5]. Under normal conditions, circulating EPCs proliferate and migrate to the injury site and develop the endothelial lining [6]. Thus, loss of bone marrow (BM)-derived hematopoietic EPCs differentiation and its subsequent lining of the blood vessels lead to cardiac dysfunction through vasoconstriction [7,8]. Consequently, maintenance of endothelial function is important in maintaining vascular homeostasis. Reports show that BM-derived EPCs incorporate into myocardial ischemic sites and also mediate neo- and revascularization after ischemic injury, thereby regulating proper blood flow to the ischemic sites [2,9-11]. However, failure in proliferation and migration of EPCs to the site of injury leads to endothelial dysfunction and disease progression. Oxidative stress leads to loss of endothelial cell function [12]. Reactive oxygen species are regulators of cellular signaling, and during oxidative stress they are involved in the deregulation of hematopoietic cell differentiation leading to endothelial dysfunction [12-15].

The antioxidant activity of rosemary extracts is attributed to carnosol and carnosic acid. Carnosol is a potent lipid peroxide and peroxyl radical scavenger [16-18]. The catechol-hydroxyl groups are regulators of the antioxidant defense mechanism through increased levels of carnosol quinone and antioxidant enzyme activities [19,20]. Carnosol induces glutathione-S-transferase (GST) activity *in vivo*, and directly interacts with electrophiles and downregulates oxidative stress [21,22]. The antiinflammatory effect of carnosol has been shown to regulate NF-κB activity, MAP kinases and proinflammatory cytokines [23,24]. Carnosol-induced anti-cancer effects have been mediated through activation of the PI3K/AKT pathway and cell cycle arrest [25,26]. In the present study, we aimed to analyze whether carnosol protects against H_2O_2-mediated inhibition of endothelial cell differentiation.

MATERIALS AND METHODS

Cell culture and effect of H_2O_2 in MAPCs proliferation

Rat bone-marrow multipotent adult progenitor cells (MAPCs) were isolated and analyzed for their marker expressions (Oct-4 and Rex-1). The cells were cultured in expansion medium at a density of $5x10^6$ cells as described [15]. The cells were then analyzed for the effect of H_2O_2 and carnosol on cellular proliferation. The cells were seeded at $1x10^5$ cells/well. H_2O_2 was added to the cells at 10-50 μM; the cells were treated for 48 h. Cell viability was determined by adding MTT after the incubation period. The absorbance was measured at 570 nm. The concentration at which cell death was induced at 50% concentration was used to analyze the protective effect of carnosol. The cells were pretreated with carnosol (0.01-0.4 μM), followed by H_2O_2 treatment. Cell viability was determined and the protective concentration was used for further studies.

Endothelial differentiation

The isolated MAPCs were cultured at a density of $5x10^6$ cells in a 25-cm^2 fibronectin-coated culture dish in the presence of 15 ng/ml VEGF as described previously [27]. The culture was monitored daily and the medium was changed every two days and analyzed for endothelial differentiation.

ROS generation and casapase-3 activity

The effect of H_2O_2 and carnosol on endothelial differentiation was monitored by evaluating ROS levels and caspase-3 activity. The cells were treated with H_2O_2 for 48 h; for identifying the protective role of carnosol, the cells were pretreated with carnosol (24 h) followed by H_2O_2 treatment. The cells were analyzed for ROS levels using DCFDA (5μg/ml for 45 mins) and the ROS generation was measured using a fluorescence spectrophotometer and represented in percentage compared to the control. For caspase-3 activity, after the above treatment the cells were allowed to differentiate and at da 10 cellular expression was analyzed through a caspase-3 activity kit (BD- Biosciences,). The results are expressed in relative activity.

Apoptotic ratio measurement

The MAPCs were treated with H_2O_2 and carnosol as per the above treatment schedule and allowed to differentiate for 10 days. After this, the cells were stained with propidium iodide (5 μg/ml) and Hoechst 33342 (5 μg/ml) and the apoptotic percentage was determined. Cells staining positive with Hoechst and PI were apoptotic or necrotic.

Antioxidant enzyme activities

After treatment, the cells were lysed and antioxidant enzyme activities GST (ab65326), GSH (ab138881), CAT (ab118184) were determined using Abcam antioxidant enzyme activity kits.

Western blot analysis

After the treatment schedule described above, the protein samples were collected and frozen in aliquots at -80°C. The proteins were separated on 15% SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membrane was blocked with 5% nonfat dried milk for 30 mins. The membrane was washed with TBST three times and incubated with primary monoclonal antibodies, Oct-4, Flk-1, CD-31, Nrf-2 (1:1000 or 1:500), at 4°C overnight. The membrane was washed with TBST three times and incubated for 1 h with secondary peroxidase-conjugated goat anti-mouse or rabbit IgG (1:10000-1:20000). After washing with TBST, the membrane was visualized by an enhanced chemiluminescence (ECL) system and densitometric analysis were performed using Image J software (GE Healthcare Life Sciences).

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, with $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ for comparisons to the control, and $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ for comparisons to H_2O_2 group. All the experiments were performed three times in triplicate to ensure reproducibility.

RESULTS AND DISCUSSION

Hematopoietic stem cell (HSC) differentiation and their involvement in endothelial function are regulated by oxidative stress. Low levels of ROS promote differentiation; however, an imbalance in ROS levels disrupts its proliferation and differentiation [28-31] In the present study, we found that treatment with H_2O_2 significantly reduced cell proliferation in a dose-dependent manner, while treatment with antioxidant carnosol protected against cell death and increased cell viability. Further, we also measured the ROS content, which was much higher during H_2O_2 treatment and potentiated apoptotic induction through increasing the apoptotic ratio and caspase-3 activity. However, pretreatment with carnosol prevented a rise in ROS levels and apoptosis by downregulating caspase-3 expression and apoptotic ratio.

First, we analyzed the effect of H_2O_2 on MAPC viability by MTT assay. H_2O_2 induced cell death (50% cell death at 27 μM). Further, we observed a protective effect of carnosol pretreatment against H_2O_2-induced cell death. Carnosol increased cell viability in a dose-dependent manner, showing maximum cell viability at a concentration of 0.2 μM in the presence of H_2O_2 (Fig. 1A, B).

Next, we identified whether carnosol protects against H_2O_2-induced oxidative stress and apoptosis in MAPCs during endothelial differentiation. H_2O_2 significantly increased the ROS levels compared to the control, while carnosol treatment significantly decreased the ROS levels compared to H_2O_2 treatment. To analyze its potential to induce apoptosis, caspase-3 activity was determined. Cells treated with H_2O_2-increased the caspase-3 activity compared to

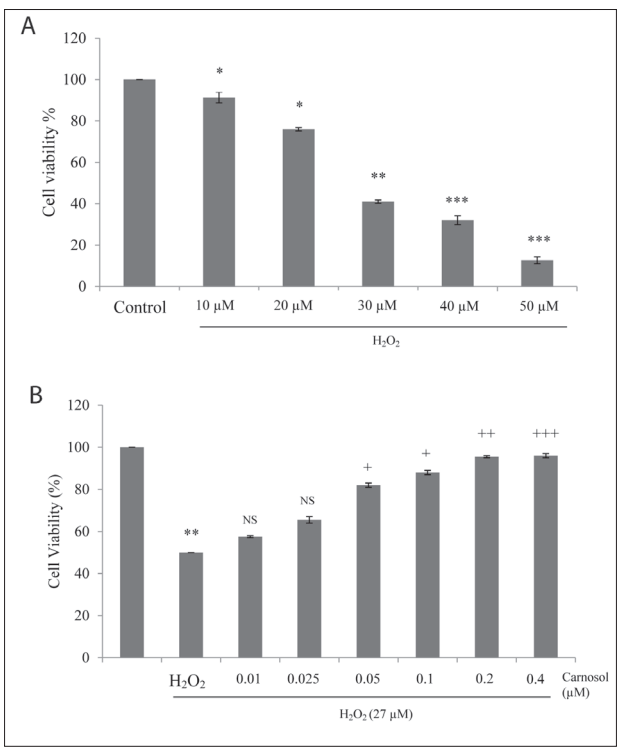

Fig. 1. Carnosol protects MAPCs against H_2O_2-induced cell death. Cell viability was determined by MTT assay. **A** – Dose-dependent cell death induced by H_2O_2 in MAPCs. **B** – Carnosol pretreatment protects against H_2O_2-induced cell death. Data represents the means±SE. One-way ANOVA followed by Tukey's multiple comparison test.

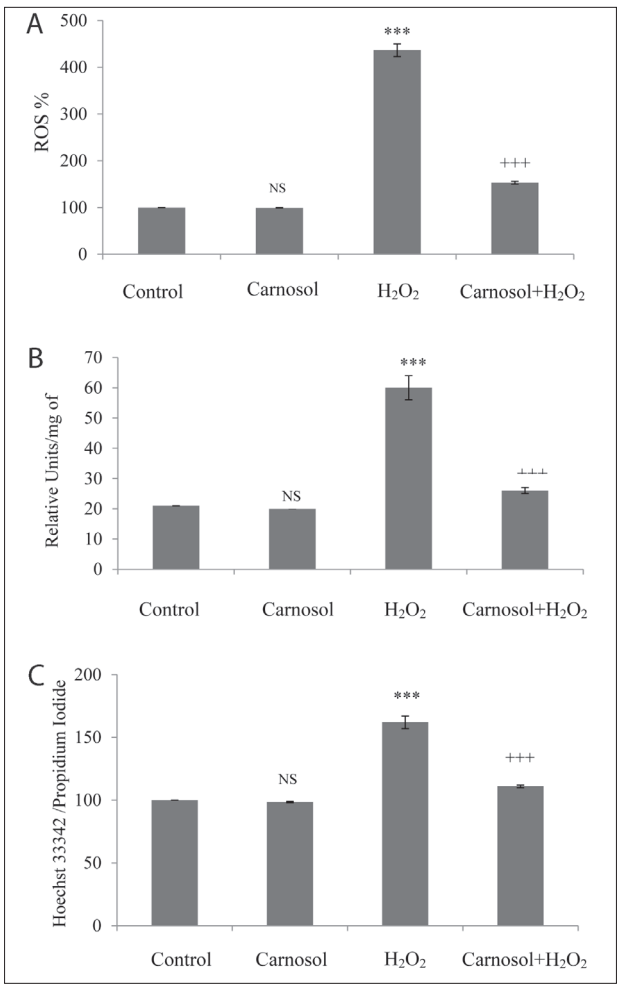

Fig. 2. Carnosol prevents H_2O_2-induced oxidative stress and apoptosis in MAPCs. **A** – ROS levels, expressed as the percentage relative compared to control cells (100%). **B** – Caspase-3 activity. **C** – Apoptotic ratio. Data represents the means±SE. One-way ANOVA followed by Tukey's multiple comparison test.

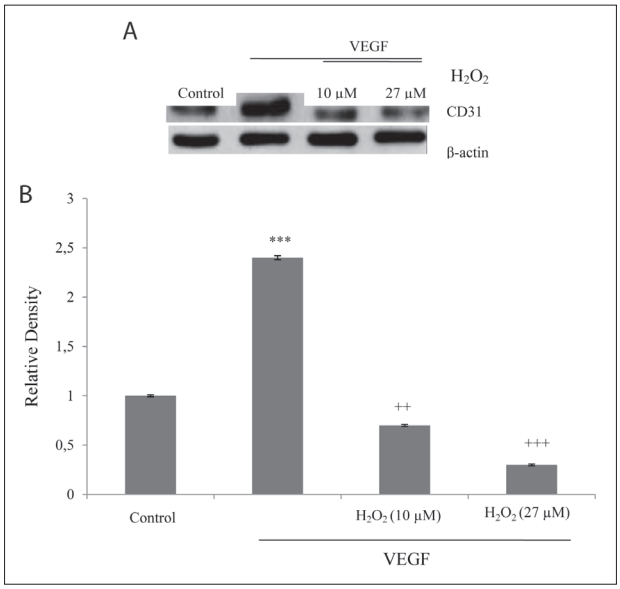

Fig 3. H_2O_2 prevents endothelial differentiation. **A** – H_2O_2 downregulates endothelial differentiation marker (CD31) in MAPCs. **B** – Relative intensity/density of CD31 expression assessed by Image J. Data represents the means±SE. One-way ANOVA followed by Tukey's multiple comparison test.

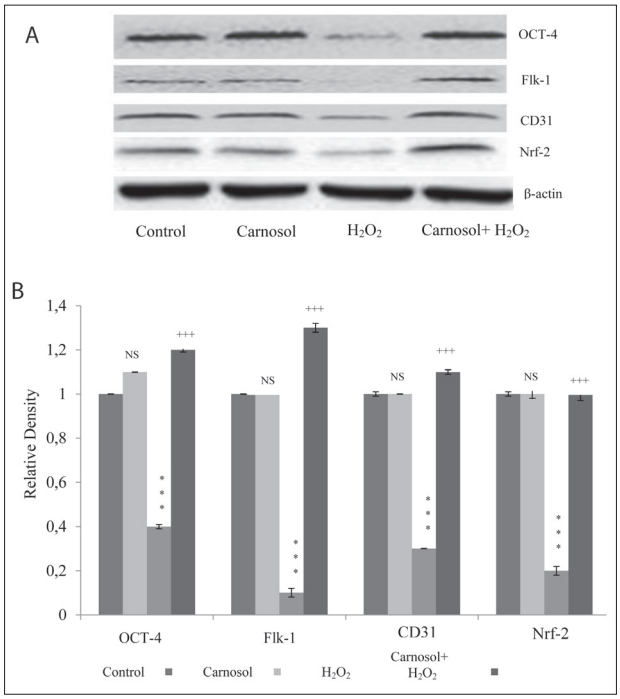

Fig. 4. Carnosol promotes endothelial differentiation under H_2O_2-induced oxidative stress in MAPCs. **A**– Examination of OCT-4, Flk-1, CD31, Nrf-2 expression by Western blotting. **B** – Relative intensity/density of protein expression assessed by Image J. Data represents the means±SE. One-way ANOVA followed by Tukey's multiple comparison test.

the control. However, carnosol pretreatment followed by H_2O_2 treatment downregulated caspase-3 activity compared to H_2O_2 alone. The apoptotic ratio significantly increased during H_2O_2 treatment compared to the control, while pretreatment with carnosol decreased the apoptotic ratio significantly compared to H_2O_2 treatment (Fig. 2A-C). The increased level of ROS during H_2O_2 treatment in mesenchymal stem cells (MSCs) observed in the present study is consistent with previous reports, and decreased the proliferation by upregulating apoptosis [15,32,33].

Transcription factor Oct-4 (octamer-binding transcription factor-4) is an embryonic stem cell marker, with Oct-4 expression determining stem cell renewal and differentiation [34-36]. Bone marrow MAPCs express increased levels of OCT-4 and have the ability to differentiate into endothelial cells [36,37]. Decreased expression of OCT-4 under oxidative stress has been previously demonstrated [38,39]. We determined the endothelial marker expression 10 days after initiation of differentiation and found upregulated expression of CD31 in VEGF treatment compared to the undifferentiated control. Treatment with H_2O_2 (10 and 27 μM) showed significant dose-dependent downregulation in CD31 expression, resulting in the inhibition of endothelial differentiation (Figs 3A, B). The expression of endothelial markers, CD31 and Flk-1 10 days after initiation of differentiation was determined. Treatment with H_2O_2 significantly downregulated OCT-4, Flk-1 and CD 31 expression compared to control cells. However, pretreatment with carnosol followed by H_2O_2 upregulated the expression of endothelial differentiation markers. Since Nrf-2 is a key protein in cellular oxidative stress; we determined its expression pattern in MAPCs. H_2O_2 treatment significantly downregulated Nrf-2 levels compared to the control, while carnosol treatment significantly increased the Nrf-2 expression compared to H_2O_2 treatment (Fig. 4A, B). Further, carnosol treatment in the presence of H_2O_2 statistically increased the antioxidant status compared to that of H_2O_2 treatment alone (Fig. 5). Our results show downregulation of OCT-4 during oxidative stress, whereas carnosol treatment increased OCT-4 expression. In addition, carnosol, as a potent antioxidant, improved the cellular Nrf-2 expression and antioxidant status and further improved the OCT-4 and endothelial marker expressions. Carnosol downregulates oxidative

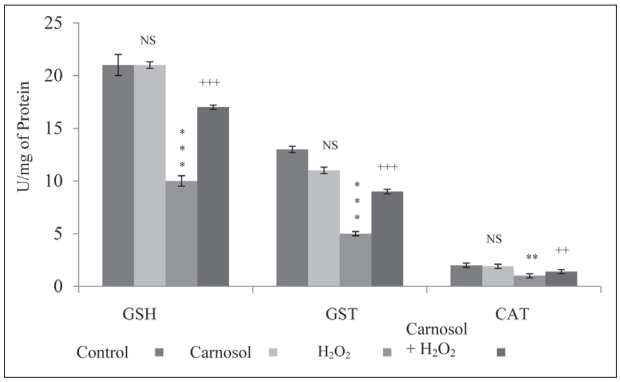

Fig. 5. Carnosol improves the antioxidant enzyme activities under H_2O_2-induced oxidative stress in MAPCs. GSH, GST and CAT activities expressed in units/mg of protein. Data represents mean±SE. One-way ANOVA followed by Tukey's multiple comparison test.

stress and inflammation through Nrf-2 upregulation [40]. Thus, carnosol regulates the redox status and improves endothelial differentiation by activating the antioxidant defense mechanism. The present study shows that redox regulation through the antioxidant carnosol supports endothelial differentiation.

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Authors' contribution: SO designed the experiments, performed the experiments, analyzed the data and prepared the manuscript. JL, LP, JZ performed the experiments and analyzed the data. LC designed the experiment, provided critical comments to the manuscript, coordinated the research and edited the write-up. All of the authors read and approved the final version of the manuscript.

Conflict of interest disclosure: The authors declare no competing interest

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