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Original Research Article

Carnosol ameliorates monosodium iodoacetate-induced osteoarthritis by targeting NF-κB and Nrf-2 in primary rat chondrocytes

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ABSTRACT

Oxidative stress and NF-κB signaling plays a major role in pathogenesis of osteoarthritis. In the present study, we analyzed the potent role of carnosol against osteoarthritis in cells treated using monosodium iodoacetate (MIA) model through *in vitro* studies. MIA caused dose-dependent cell death and induced programmed cell death by increasing subG1 accumulation and caspase-3 expressions. MIA caused oxidative stress by increasing reactive oxygen species, lipid peroxidation and further induced NF-κB expression and down regulated Nrf-2 levels. Pre-treatment with carnosol significantly protected the cells by reducing the oxidative stress markers and improved the cell viability up to 98%. Further, carnosol down regulated NF-κB nuclear expression with a concomitant increase in Nrf-2 nuclear localization and up regulated the nuclear Nrf-2 levels. Carnosol also inhibited MIA-induced subG1 accumulation and caspase-3 activation. This study demonstrates that, carnosol might act as potent antioxidant and regulate MIA-induced oxidative stress, NF-κB signaling and programmed cell death by up regulating the Nrf-2 levels.

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Introduction

Articular cartilage constitutes chondrocytes, proteoglycans, collagen along with various non-collagen proteins. The chondrocytes play an important role in the maintenance of extracellular matrix through renewal of matrix proteins. However, several etiologies related to shift in equilibrium and functional changes includes obesity, trauma, mechanical injury, and other systemic diseases (Goldring and Goldring, 2007). Oxidative stress is one of the central regulators in the development of osteoarthritis. Immune activation and release of inflammatory cytokines contributes to the pathogenesis of osteoarthritis (Henrotin et al., 2003; Li et al., 2012). Monosodium iodoacetate (MIA) model mediates apoptotic cell death in chondrocytes and induces degradation in cartilage tissues *in vitro* and *in vivo* (Grossin et al., 2006; Ivanavicius et al., 2007). Chondrocyte function and viability is closely related in the development and progression of osteoarthritis. NF-κB and MAPK-Erk mediated molecular signaling in chondrocytes inhibit collagen synthesis and involve in cartilage damage and destruction. Pro-inflammatory cytokines IL-1β and TNF-α also activates NF-κB and further potentiates the cartilage loss (Dossumbekova et al., 2007; Loeser et al., 2003; Ronzière et al., 2005). Chondrocyte stress response and chronic inflammation are linked to hypertrophy and apoptosis (Aigner et al., 2004; Clegg and Mobasheri, 2003; Healy et al., 2005).

Development of chondrocyte dysfunction is mediated through redox signaling, thus significant protection strategy could be mediated through antioxidant treatment. In the present study, we aimed at testing carnosol against MIA-induced redox regulation in rat chondrocytes. Carnosol, ortho-diphenolic diterpene, polyphenol found in rosemary and Sage with higher antioxidant and anti-inflammatory property (Aruoma et al., 1992; Lo et al., 2002). Reports also suggest the anti-cancer potential of carnosol against prostrate, breast, skin cancers (Huang et al., 1994; Johnson et al., 2008; Singletary et al., 1996; Vergara et al., 2014; Valdés et al., 2014). However, its effect on osteoarthritis has not been explored. In this study, we have analyzed the cytotoxic potential of MIA and protective effect offered by carnosol against these effects. Further, mechanism involved in protection offered by carnosol on MIA-induced oxidative stress, inflammation and programmed cell death were studied.

Materials and methods

Primary cell culture and treatment

The primary rat chondrocyte isolation and culturing procedure was followed as previously reported with minor modifications (Jiang et al., 2013). Five-weeks-old Sprague-Dawley rats were used for the present study. The articular cartilages from femoral heads were isolated and digested in trypsin–EDTA for 30 min and then treated with collagenase (0.2%) solution for 3 h. The chondrocytes isolated were cultured in Minimal Essential Eagel's Medium supplemented with 10% FCS and 1% pencillin and streptomycin (5% CO_2, 37 °C). MIA and carnosol were dissolved in sterile distilled water and DMSO respectively and stock solutions were prepared at 1 M concentration.

Cell viability

MIA-induced cell death in rat chondrocytes were tested at different concentrations using MTT assay (Mosmann, 1983). For this, cells (1×10^5) were seeded in 96 well plates and allowed for 1 h attachment. In the preliminary study we have performed MIA-induced cell death at 2, 6, 12 and 24 h and carnosol pre-treatment for 12 and 24 h. However, only 24 h treatment at different concentration of MIA showed dose dependent cell death during MIA treatment. Further, carnosol treatment at 12 h did not show cytoprotection (data not shown). So further studies were continued at 24 h time point. Further, maximum volume of vehicle used in the study was tested for the cellular toxicity. The results showed that vehicle by itself did not induce any cell death. The cells were treated with different concentration of MIA from 2 to 12 μM concentration for 24 h. For cytoprotection studies, carnosol (2–10 μM) was treated for 24 h, followed by MIA. The medium was removed after treatments and MTT (Cat. M5655, Sigma) was added and incubated for another 5 h, followed which dissolved in DMSO (472301, Sigma) and absorbance was read in spectrophotometer at 570 nm. All the groups were performed in triplicates under 3 independent experiments and the results were expressed as percentage cell viability compared to the control.

Reactive oxygen species

Cellular reactive oxygen species were determined using DCFDA – Cellular Reactive Oxygen Species Detection Assay Kit (ab113851). For this, cells (1×10^6) were plated in 6 well plate and incubated with 10 μM MIA for 24 h. For identifying the protective role of carnosol; 10 μM carnosol was treated for 24 h. DCFDA solution was incubated for 45 min. Followed by which the cells were treated with MIA for 24 h. Carnosol treatment and control was also kept as separate group. The reading was taken in fluorometer (Ultrospec 3000, Pharmacia Biotech, Milan) and changes in fluorescence intensity at Ex: 485 and Em: 535 were recorded.

Lipid peroxide content

The cells (1×10^6) were treated with 10 μM carnosol for 24 h followed by 10 μM MIA for 24 h. Separate groups of carnosol, MIA and control were maintained. The lipid peroxide content was determined as described in Lipid Peroxidation (MDA) Assay Kit (ab118970).

Western blot analysis

The cells (1×10^6) were treated with 10 μM carnosol for 24 h followed by 10 μM MIA for 24 h. For carnosol alone or MIA alone treatment, cells were treated with respective compounds for 24 h. The protein expressions were determined through western blot. Cytosolic and nuclear extracts were isolated using Nuclear/Cytosolic Fractionation Kit (Cellbiolabs, Inc.) from different groups after treatment schedule. The 50 μg

of isolated protein were separated in 12% SDS-PAGE and transferred into nitrocellulose membrane. The blots were blocked with 5% skimmed milk for 1 h and probed with primary antibodies against (NF-κB, Abcam, 1:1000) and (Nrf-2, Abcam, 1:1000) for overnight. After which, the blots were washed with TBST thrice and anti-mouse secondary antibodies (Abcam, 1:10,000) were added for 1 h. The blots were washed with TBST thrice and developed with enhanced chemiluminescence system and analyzed using ImageJ software (Rasband, http://imagej.nih.gov/ij/, 1997–2015). β-Actin and GAPDH was used as internal control.

Nuclear localization study

The Nrf-2 nuclear localization in carnosol and MIA treated rat chondrocytes were determined through immunofluorescence. Fluorescent labeled antibodies were used to detect the location of proteins either in cytoplasm or nucleus; while the nucleus was counter stained with DAPI (D9542, Sigma). The cells (5×10^5) were grown in cover slips and treated with 10 μM carnosol for 24 h followed by 10 μM MIA for 24 h. For carnosol alone or MIA alone treatment, cells were treated with respective compounds for 24 h. After treatment schedule, the cells were treated with primary antibody and FITC conjugated secondary antibody. The nucleus was stained with DAPI for 5 min. The images were ImageJ software.

Cell cycle analysis

The cells (1×10^6) were treated with 10 μM carnosol for 24 h followed by 10 μM MIA for 24 h. For carnosol alone or MIA alone treatment, cells were treated with respective compounds for 24 h. After the treatment schedule, the cells were washed with PBS twice and stained with 1 μg of propidium iodide (P4170, Sigma) stain for 30 min and apoptotic induction (subG1 accumulation) was analyzed for using BD FACSVerse and the data was processed with ModFit LT – premiere DNA cell-cycle analysis software.

Caspase-3 activity

The cells (1×10^6) were treated with 10 μM carnosol for 24 h followed by 10 μM MIA for 24 h. For carnosol alone or MIA alone treatment, cells were treated with respective compounds for 24 h. The cells after respective treatments were

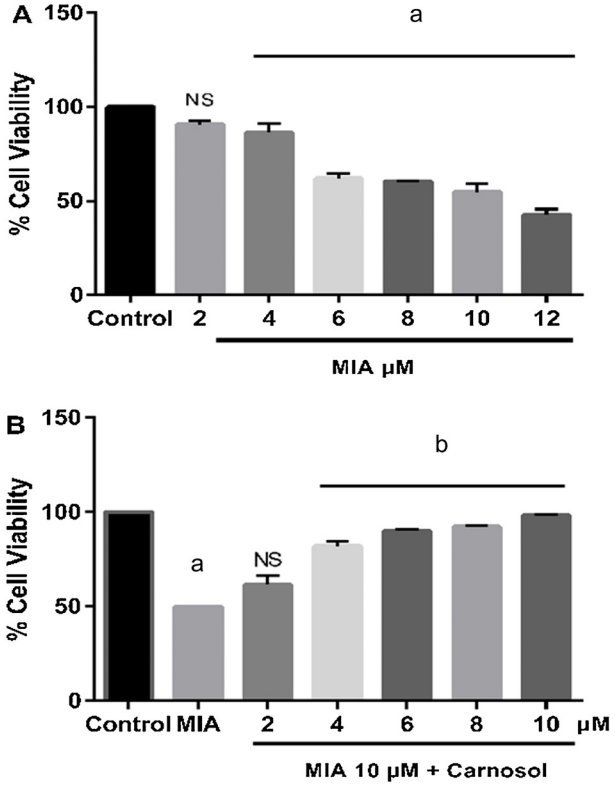

Fig. 1 – Carnosol increases cell viability against MIA-induced chondrocyte cell death. Cell viability was measured by MTT assay. (A) MIA-induced dose-dependent chondrocyte cell death. (B) Cytoprotective effect of carnosol against MIA-induced cell death. Expressed in percentage compared to control (100%). Data are represented in mean ± SEM. (a) Statistically significant; NS, non-significant when compared to control group. (b) Statistically significant; NS, non-significant when compared to MIA group (one way ANOVA followed by Tukey's multiple comparison).

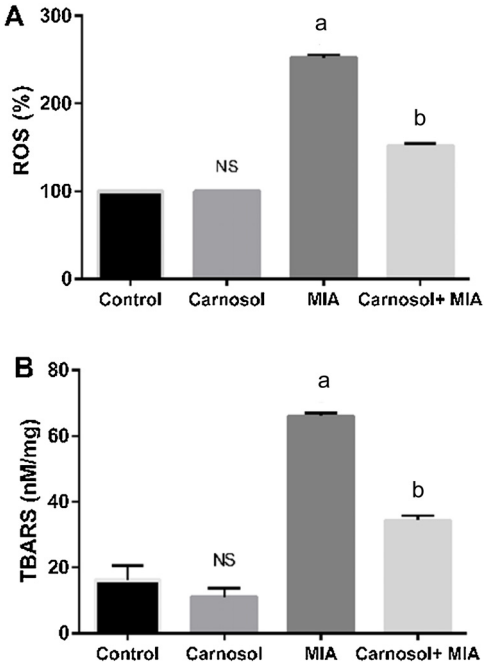

Fig. 2 – Carnosol inhibits MIA-mediated ROS and LPO levels. (A) ROS expressed as % Fluorescence compared to control. (B) Lipid peroxide expressed as TBARS/mg of protein (nM). Data are represented in mean ± SEM. (a) Statistically significant; NS, non-significant when compared to control group. (b) Statistically significant; NS, non-significant when compared to MIA group (one way ANOVA followed by Tukey's multiple comparison).

analyzed for caspase-3 activity using Caspase 3 Assay Kit, Fluorimetric (Sigma). The results were expressed in relative density/mg of protein.

Statistical analysis

All the experiments were performed as 3 independent experiments in triplicates. The data were represented as mean ± SE. Statistical analysis was performed with one way ANOVA – followed by two Tukey's multiple comparison test, at the significance level alpha = 0.05.

Results

Carnosol protects against MIA-induced cell death in rat chondrocytes

Results from the cell viability assay shows that, MIA caused dose dependent cell death and IC_{50} concentration was found to be 10 μM (Fig. 1A). Further, cytoprotective concentration of carnosol was performed by pre-treatment studies. The cells were treated with different concentration of carnosol for 24 h followed by MIA treatment. Concentration at 10 μM showed highest cytoprotection against MIA-induced cell death compared to that of MIA treated cells. The cell viability was found to around 98%. So, we selected this particular dose to understand the detailed molecular mechanism (Fig. 1B).

Carnosol prevents MIA-induced ROS and LPO levels

In order to understand the role of carnosol on oxidative stress, reactive oxygen species and LPO levels were estimated (Fig. 2). MIA (10 μM) caused a significant increase in oxidative markers compared to control cells. However, pre-treatment with carnosol (10 μM) followed by MIA treatment showed significant decline in the levels compared to that MIA treated cells.

Carnosol prevents NF-κB expression and induces Nrf-2 levels

We next analyzed the expressions of cytosolic and nuclear levels of redox transcription factors during MIA and carnosol treatment. MIA treatment up regulated the nuclear levels of NF-κB with concomitant down expression in cytosol levels. Further, the regulatory protein of antioxidant defense system Nrf-2 levels was significantly down regulated in the nucleus by MIA treatment. Pre-treatment with carnosol followed by MIA treatment down regulated the nuclear NF-κb levels with induction of Nrf-2 expression compared to MIA treatment (Fig. 3).

Carnosol induces Nrf-2 nuclear localization

Nrf-2 levels in the nucleus under different treatment schedules were analyzed by immunofluorescence technique. Fig. 4 shows that carnosol treatment and pre-treatment with carnosol followed by MIA treatment showed Nrf-2 nuclear localization. While MIA treatment alone did not induce Nrf-2

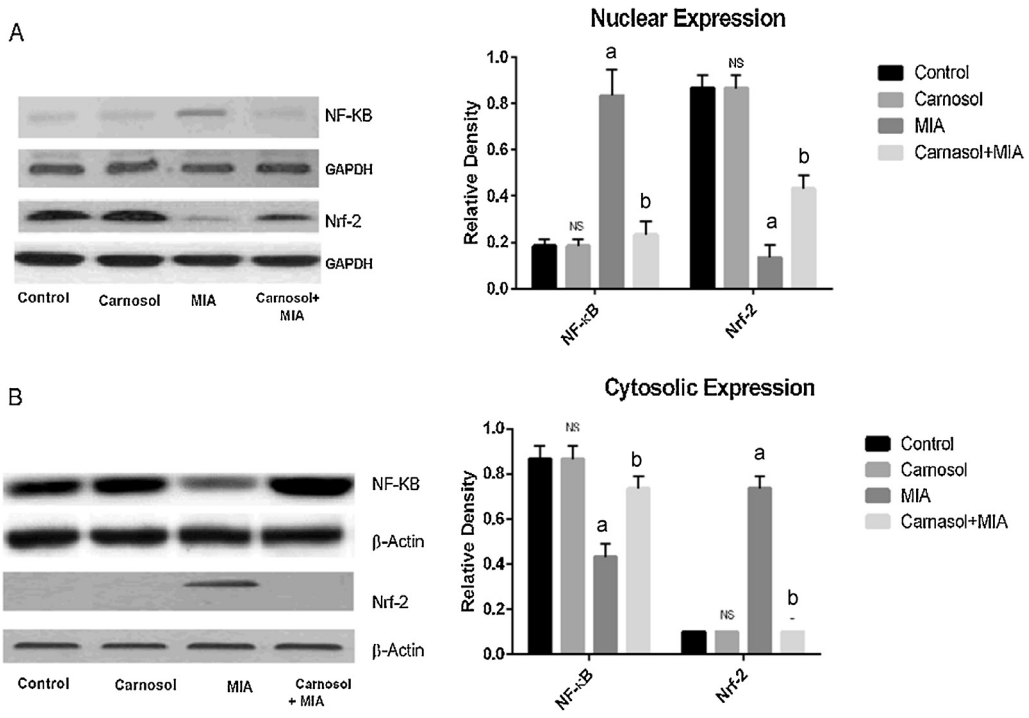

Fig. 3 – Carnosol induces Nrf-2 and down regulates NF-κB expressions. Effect of carnosol and MIA treatment on. (A). NF-κb and Nrf-2 nuclear expression. (B) NF-κb and Nrf-2 cytosolic expression. The blots are quantified using ImageJ software and relative levels were expressed compared to internal control β-actin or GAPDH. Data are represented in mean ± SEM. (a) Statistically significant; NS, non-significant when compared to control group. (b) Statistically significant; NS, non-significant when compared to MIA group (one way ANOVA followed by Tukey's multiple comparison).

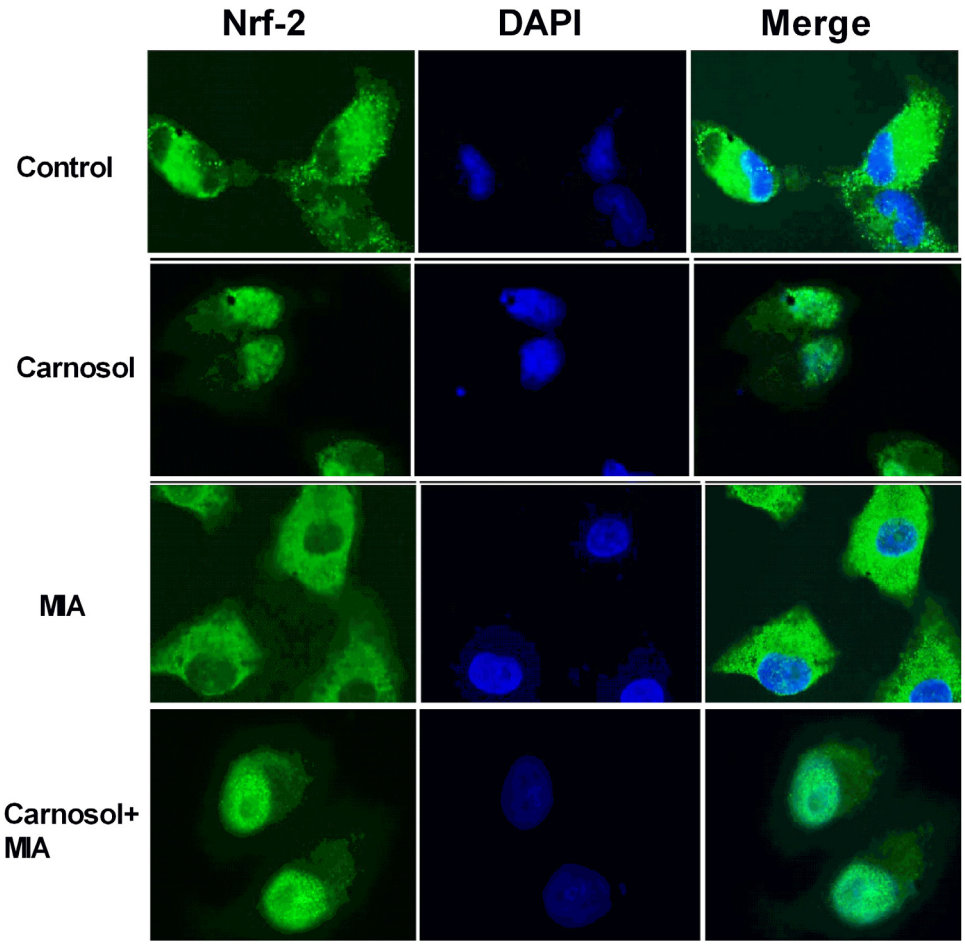

Fig. 4 – Carnosol induces Nrf-2 nuclear localization. Immunofluorescence of Nrf-2 was analyzed in MIA and carnosol treated cells. Nrf-2 was detected using FITC conjugated secondary antibody and nucleus was stained with DAPI.

nuclear localization. These results show that carnosol treatment activates Nrf-2 levels in the nucleus and protects the cells against toxic insults.

Carnosol prevents programmed cell death in rat chondrocytes

Fig. 5 shows that MIA treatment induced subG1 accumulation with up regulated levels of caspase-3 compared to that of control cells. Pre-treatment with carnosol and then treated with MIA prevented MIA induced programmed cell death by reducing the subG1 accumulation and caspase-3 activity.

Discussion

Chondrocytes regulate articular cartilage homeostasis; however deregulated homeostasis with activation of redox signaling and chronic inflammation leads to development of osteoarthritis (Goldring and Marcu, 2009). In the present study, we sought to identify whether antioxidant carnosol might protect against osteoarthritis *in vitro* by regulating redox signaling.

Oxidative stress is an important contributing factor in pathogenesis of osteoarthritis. Reactive oxygen species promote cartilage destruction in osteoarthritis and induces chronic inflammation. Under oxidative stress, cartilage tissues shows elevated levels of proteolytic enzymes leading to an equilibrium shift to catabolic processes with the destruction of cartilage (Goldring and Marcu, 2009; Canter et al., 2007). In the present study, we found MIA-induced cell death in chondrocytes in a dose dependent manner. MIA also caused severe oxidative stress by increasing reactive oxygen species levels and lipid peroxidation. Carnosol pre-treatment significantly reduced the MIA-induced oxidative stress and improved the cell viability, thus preventing MIA-induced cellular stress. Previous studies have been demonstrated on antioxidant property of carnosol through inhibition of LDL oxidation and lipid free radicals (Aruoma et al., 1992; Zeng et al., 2001).

NF-κB signaling is linked to pathogenesis of osteoarthritis. Transcription factor, NF-κB once activated under redox signaling translocates into the nucleus and regulates target gene inflammatory gene transcription. NF-κB mediated inflammation in chondrocytes induces damage to the extracellular matrix, leading to cartilage destruction and apoptosis. TNF-α induces ICAM-1, collagenase and downstream cytokine expressions and further potentiates NF-κB mediated responses in osteoarthritis (Largo et al., 2003; Lianxu et al., 2006; Aupperle et al., 2001; Roman-Blas and Jimenez, 2006).

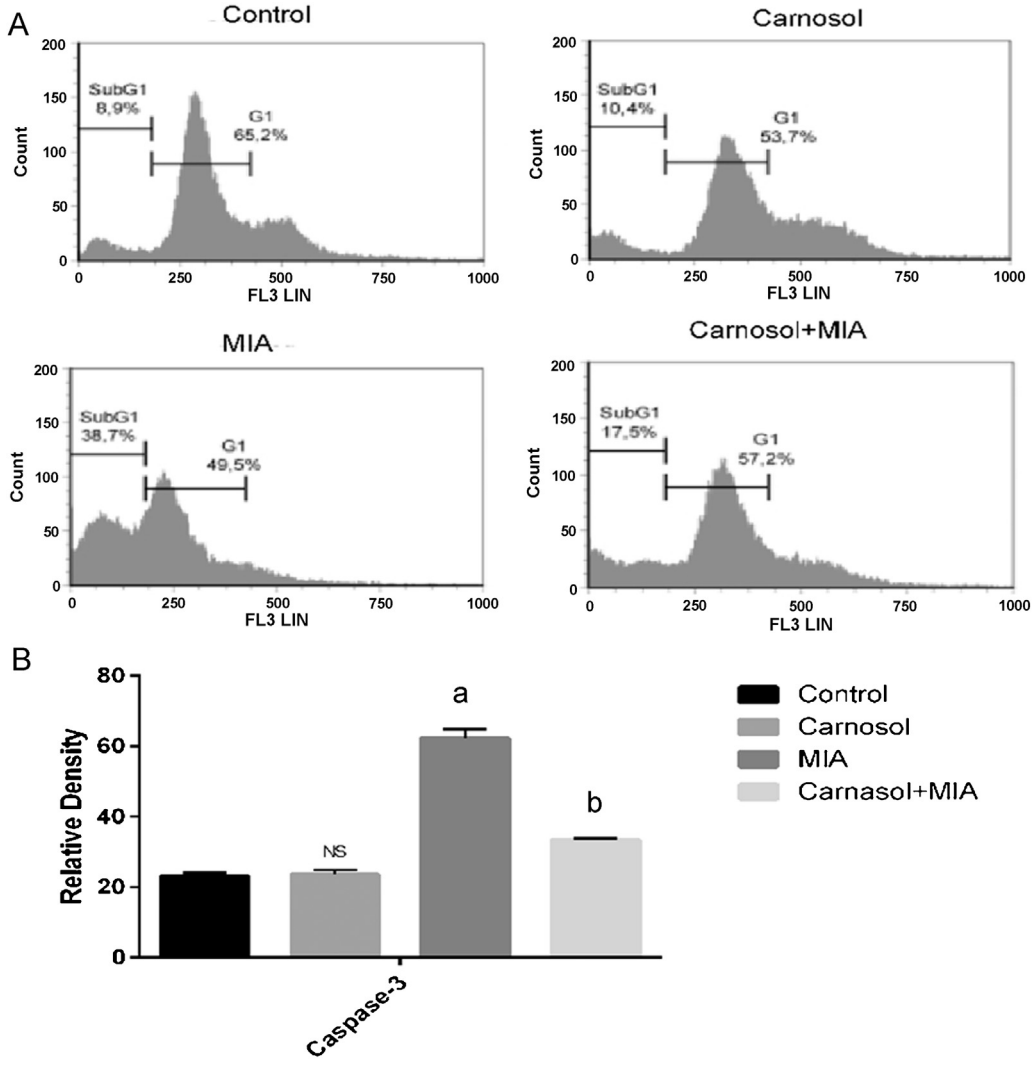

Fig. 5 – MIA-induced programmed cell death was prevented by carnosol. (A) FACS analysis of SubG1 accumulation. (B) Casapase-3 expressions were analyzed by ELISA. Data are represented in mean ± SEM. (a) Statistically significant; NS, non-significant when compared to control group. (b) Statistically significant; NS, non-significant when compared to MIA group (one way ANOVA followed by Tukey's multiple comparison).

Significant up regulation of nuclear NF-κB expressions with concomitant down regulation of cytoplasmic levels of NF-KB was observed during MIA treatment. On pre-treatment with antioxidant carnosol, the nuclear NF-κB levels were down regulated. Lian et al. (2010) reported protective role of carnosol against TNF-α induced endothelial cells through inhibition of NF-κB and up regulation HO-1 expression. Anti-inflammatory effect of carnosol has been reported through inhibition of LPS-induced NO and NF-κB in mouse macrophages (Lo et al., 2002). Carnosol mediated anti-inflammation through down regulation of inflammatory cytokines in human polymorphonuclear leukocytes and amelioration of leukocyte infiltration in TPA-induced mouse ear edema (Mengoni et al., 2011; Poeckel et al., 2008).

Nrf-2 is another nuclear transcription factor responds under oxidative stress conditions and ameliorates redox signaling by regulating antioxidant balance inside the cells. Keap1 (Kelch-like ECH-associated protein 1) is associated with Nrf-2 in the cytoplasm and regulates its ubiquitination. During oxidative stress, covalent modification of Keap-1-SH groups results in Nrf-2 release and migration into nucleus (Kobayashi et al., 2006). Belonging to ''cap 'n' collar'' family of basic leucine zipper transcription factors, activated Nrf-2 translocates into the nucleus and regulates the expression of 250 genes among them antioxidant enzymes, including, NAD (P) H quinine oxidoreductase 1, glutathione-S-transferases, heme oxygenase 1 (HO-1) (Li et al., 2008; Wakabayashi et al., 2010). In our study, we found that, MIA treatment lead to decline in Nrf-2 levels while pre-treatment with carnosol increased the Nrf-2 expressions showing cytoprotective role against osteoarthritis. In addition, carnosol induced nuclear translocation of Nrf-2 while MIA treatment retained the Nrf-2 in the cytoplasm. The results show evidence that carnosol regulates oxidative imbalance by Nrf-2 activation and expression against MIA-induced response. These observations are consistent with previous studies where, decline in Nrf-2 responses occurs

during deregulated oxidative stress conditions (Ungvari et al., 2011; Tomobe et al., 2012). The protective role of carnosol by enhancing Nrf-2 and antioxidant enzymes have been demonstrated in earlier studies (Martin et al., 2004; Chen et al., 2011).

Cartilage degradation in osteoarthritis is associated with chondrocyte cell death. Pathogenesis and disease severity is associated with a decline in chondrocyte number, possibly through oxidative stress, decline in growth factors, proteoglycans depletion (Thomas et al., 2007; Hashimoto et al., 1998). Chondrocyte apoptosis during osteoarthritis is well studied (Sharif et al., 2004; Almonte-Becerril et al., 2010; Taniguchi et al., 2012). Sub-G1 accumulation by FACS analysis and significant up-regulation of caspase-3 expressions show that MIA-induced programmed cell death in chondrocytes. We identified that carnosol significantly reduced MIA-induced programmed cell death by decreasing caspase-3 expressions and sub-G1accumulation. Protective role of carnosol against rotenone-induced neurotoxicity was mediated through down regulation of caspase-3 levels (Kim et al., 2006). Carnosol induced cytoprotection against SNP-mediated cell death was mediated through modulation of antioxidant HO-1 levels and apoptotic events (Kim et al., 2010).

Our findings show that carnosol as protective antioxidant against MIA-induced oxidative stress and programmed cell death by suppressing NF-κB and activating Nrf-2 transcription factor.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Aigner, T., Kim, H.A., Roach, H.I., 2004. Apoptosis in osteoarthritis. Rheum. Dis. Clin. 30, 639–653.

Almonte-Becerril, M., Navarro-Garcia, F., Gonzalez-Robles, A., Vega-Lopez, M.A., Lavalle, C., Kouri, J.B., 2010. Cell death of chondrocytes is a combination between apoptosis and autophagy during the pathogenesis of osteoarthritis within an experimental model. Apoptosis 15, 631–638.

Aruoma, O.I., Halliwell, B., Aeschbach, R., Löligers, J., 1992. Antioxidant and pro-oxidant properties of active rosemary constituents: carnosol and carnosic acid. Xenobiotica 22, 257–268.

Aupperle, K.R., Bennett, B.L., Han, Z., Boyle, D.L., Manning, A.M., Firestein, G.S., 2001. NF-κB regulation by IκB kinase-2 in rheumatoid arthritis synoviocytes. J. Immunol. 166, 2705–2711.

Canter, P.H., Wider, B., Ernst, E., 2007. The antioxidant vitamins A, C, E and selenium in the treatment of arthritis: a systematic review of randomized clinical trials. Rheumatology 46, 1223–1233.

Chen, C.-C., Chen, H.-L., Hsieh, C.-W., Yang, Y.-L., Wung, B.-S., 2011. Upregulation of NF-E2-related factor-2-dependent glutathione by carnosol provokes a cytoprotective response and enhances cell survival. Acta Pharmacol. Sin. 32, 62–69.

Clegg, P.D., Mobasheri, A., 2003. Chondrocyte apoptosis, inflammatory mediators and equine osteoarthritis. Vet. J. 166, 3–4.

Dossumbekova, A., Anghelina, M., Madhavan, S., He, L., Quan, N., Knobloch, T., Agarwal, S., 2007. Biomechanical signals inhibit IKK activity to attenuate NF-κB transcription activity in inflamed chondrocytes. Arthritis Rheum. 56, 3284–3296.

Goldring, M.B., Goldring, S.R., 2007. Osteoarthritis. J. Cell. Physiol. 213, 626–634.

Goldring, M.B., Marcu, K.B., 2009. Cartilage homeostasis in health and rheumatic diseases. Arthritis Res. Ther. 11, 224.

Grossin, L., Cournil-Henrionnet, C., Pinzano, A., Gaborit, N., Dumas, D., Etienne, S., Stoltz, J.F., Terlain, B., Netter, P., Mir, L. M., Gillet, P., 2006. Gene transfer with HSP 70 in rat chondrocytes confers cytoprotection in vitro and during experimental osteoarthritis. FASEB J. 20, 65–75.

Hashimoto, S., Ochs, R.L., Komiya, S., Lotz, M., 1998. Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. Arthritis Rheum. 41, 1632–1638.

Healy, Z.R., Lee, N.H., Gao, X., Goldring, M.B., Talalay, P., Kensler, T.W., Konstantopoulos, K., 2005. Divergent responses of chondrocytes and endothelial cells to shear stress: cross-talk among COX-2, the phase 2 response, and apoptosis. Proc. Natl. Acad. Sci. U. S. A. 102, 14010–14015.

Henrotin, Y.E., Bruckner, P., Pujol, J.P.L., 2003. The role of reactive oxygen species in homeostasis and degradation of cartilage. Osteoarthr. Cartil. 11, 747–755.

Huang, M.-T., Ho, C.-T., Wang, Z.Y., Ferraro, T., Lou, Y.-R., Stauber, K., Ma, W., Georgiadis, C., Laskin, J.D., Conney, A.H., 1994. Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. Cancer Res. 54, 701–708.

Ivanavicius, S.P., Ball, A.D., Heapy, C.G., Westwood, R.F., Murray, F., Read, S.J., 2007. Structural pathology in a rodent model of osteoarthritis is associated with neuropathic pain: increased expression of ATF-3 and pharmacological characterisation. Pain 128, 272–282.

Jiang, L., Li, L., Geng, C., Gong, D., Jiang, L., Ishikawa, N., Kajima, K., Zhong, L., 2013. Monosodium iodoacetate induces apoptosis via the mitochondrial pathway involving ROS production and caspase activation in rat chondrocytes in vitro. J. Orthop. Res. 31, 364–369.

Johnson, J.J., Syed, D.N., Heren, C.R., Suh, Y., Adhami, V.M., Mukhtar, H., 2008. Carnosol, a dietary diterpene, displays growth inhibitory effects in human prostate cancer PC3 cells leading to G2-phase cell cycle arrest and targets the 5′-AMP-activated protein kinase (AMPK) pathway. Pharm. Res. 25, 2125–2134.

Kim, S.-J., Kim, J.-S., Cho, H.-S., Lee, H.J., Kim, S.Y., Kim, S., Lee, S.-Y., Chun, H.S., 2006. Carnosol, a component of rosemary (*Rosmarinus officinalis* L.) protects nigral dopaminergic neuronal cells. NeuroReport 17, 1729–1733.

Kim, S.Y., Park, E., Park, J.A., Choi, B.-S., Kim, S., Jeong, G., Kim, C.-S., Kim, D.K., Kim, S.-J., Chun, H.S., 2010. The plant phenolic diterpene carnosol suppresses sodium nitroprusside-induced toxicity in C6 glial cells. J. Agric. Food Chem. 58, 1543–1550.

Kobayashi, A., Kang, M.-I., Watai, Y., Tong, K.I., Shibata, T., Uchida, K., Yamamoto, M., 2006. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. Mol. Cell. Biol. 26, 221–229.

Largo, R., Alvarez-Soria, M.A., Díez-Ortego, I., Calvo, E., Sánchez-Pernaute, O., Egido, J., Herrero-Beaumont, G., 2003. Glucosamine inhibits IL-1β-induced NFκB activation in human osteoarthritic chondrocytes. Osteoarthr. Cartil. 11, 290–298.

Li, W., Khor, T.O., Xu, C., Shen, G., Jeong, W.-S., Yu, S., Kong, A.-N., 2008. Activation of Nrf2-antioxidant signaling attenuates NF-κB-inflammatory response and elicits apoptosis. Biochem. Pharmacol. 76, 1485–1489.

Li, D., Xie, G., Wang, W., 2012. Reactive oxygen species: the 2-edged sword of osteoarthritis. Am. J. Med. Sci. 344, 486–490.

Lian, K.-C., Chuang, J.-J., Hsieh, C.-W., Wung, B.-S., Huang, G.-D., Jian, T.-Y., Sun, Y.-W., 2010. Dual mechanisms of NF-κB inhibition in carnosol-treated endothelial cells. Toxicol. Appl. Pharmacol. 245, 21–35.

Lianxu, C., Hongti, J., Changlong, Y., 2006. NF-κBp65-specific siRNA inhibits expression of genes of COX-2, NOS-2 and MMP-9 in rat IL-1β-induced and TNF-α-induced chondrocytes. Osteoarthr. Cartil. 14, 367–376.

Lo, A.-H., Liang, Y.-C., Lin-Shiau, S.-Y., Ho, C.-T., Lin, J.-K., 2002. Carnosol, an antioxidant in rosemary, suppresses inducible nitric oxide synthase through down-regulating nuclear factor-κB in mouse macrophages. Carcinogenesis 23, 983–991.

Loeser, R.F., Forsyth, C.B., Samarel, A.M., Im, H.-J., 2003. Fibronectin fragment activation of proline-rich tyrosine kinase PYK2 mediates integrin signals regulating collagenase-3 expression by human chondrocytes through a protein kinase C-dependent pathway. J. Biol. Chem. 278, 24577–24585.

Martin, D., Rojo, A.I., Salinas, M., Diaz, R., Gallardo, G., Alam, J., de Galarreta, C.M.R., Cuadrado, A., 2004. Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. J. Biol. Chem. 279, 8919–8929.

Mengoni, E.S., Vichera, G., Rigano, L.A., Rodriguez-Puebla, M.L., Galliano, S.R., Cafferata, E.E., Pivetta, O.H., Moreno, S., Vojnov, A.A., 2011. Suppression of COX-2, IL-1β and TNF-α expression and leukocyte infiltration in inflamed skin by bioactive compounds from *Rosmarinus officinalis* L. Fitoterapia 82, 414–421.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Poeckel, D., Greiner, C., Verhoff, M., Rau, O., Tausch, L., Hörnig, C., Steinhilber, D., Schubert-Zsilavecz, M., Werz, O., 2008. Carnosic acid and carnosol potently inhibit human 5-lipoxygenase and suppress pro-inflammatory responses of stimulated human polymorphonuclear leukocytes. Biochem. Pharmacol. 76, 91–97.

Rasband, W.S., 1997–2015. ImageJ. U. S. National Institutes of Health, Bethesda, MD, USA. , http://imagej.nih.gov/ij/.

Roman-Blas, J.A., Jimenez, S.A., 2006. NF-κB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. Osteoarthr. Cartil. 14, 839–848.

Ronzière, M.-C., Aubert-Foucher, E., Gouttenoire, J., Bernaud, J., Herbage, D., Mallein-Gerin, F., 2005. Integrin α1β1 mediates collagen induction of MMP-13 expression in MC615 chondrocytes. Biochim. Biophys. Acta 1746, 55–64.

Sharif, M., Whitehouse, A., Sharman, P., Perry, M., Adams, M., 2004. Increased apoptosis in human osteoarthritic cartilage corresponds to reduced cell density and expression of caspase-3. Arthritis Rheum. 50, 507–515.

Singletary, K., MacDonald, C., Wallig, M., 1996. Inhibition by rosemary and carnosol of 7,12-dimethylbenz[a]anthracene (DMBA)-induced rat mammary tumorigenesis and in vivo DMBA–DNA adduct formation. Cancer Lett. 104, 43–48.

Taniguchi, S., Ryu, J., Seki, M., Sumino, T., Tokuhashi, Y., Esumi, M., 2012. Long-term oral administration of glucosamine or chondroitin sulfate reduces destruction of cartilage and up-regulation of MMP-3 mRNA in a model of spontaneous osteoarthritis in Hartley guinea pigs. J. Orthop. Res. 30, 673–678.

Thomas, C.M., Fuller, C.J., Whittles, C.E., Sharif, M., 2007. Chondrocyte death by apoptosis is associated with cartilage matrix degradation. Osteoarthr. Cartil. 15, 27–34.

Tomobe, K., Shinozuka, T., Kuroiwa, M., Nomura, Y., 2012. Age-related changes of Nrf2 and phosphorylated GSK-3β in a mouse model of accelerated aging (SAMP8). Arch. Gerontol. Geriatr. 54, e1–e7.

Ungvari, Z., Bailey-Downs, L., Sosnowska, D., Gautam, T., Koncz, P., Losonczy, G., Ballabh, P., de Cabo, R., Sonntag, W.E., Csiszar, A., 2011. Vascular oxidative stress in aging: a homeostatic failure due to dysregulation of NRF2-mediated antioxidant response. Am. J. Physiol. 301, H363–H372.

Valdés, A., García-Cañas, V., Simó, C., Ibáñez, C., Micol, V., Ferragut, J.A., Cifuentes, A., 2014. Comprehensive foodomics study on the mechanisms operating at various molecular levels in cancer cells in response to individual rosemary polyphenols. Anal. Chem. 86, 9807–9815.

Vergara, D., Simeone, P., Bettini, S., Tinelli, A., Valli, L., Storelli, C., Leo, S., Santino, A., Maffia, M., 2014. Antitumor activity of the dietary diterpene carnosol against a panel of human cancer cell lines. Food Funct. 5, 1261–1269.

Wakabayashi, N., Slocum, S.L., Skoko, J.J., Shin, S., Kensler, T.W., 2010. When NRF2 talks, who's listening? Antioxid. Redox Signal. 13, 1649–1663.

Zeng, H.H., Tu, P.F., Zhou, K., Wang, H., Wang, B.H., Lu, J.F., 2001. Antioxidant properties of phenolic diterpenes from *Rosmarinus officinalis*. Acta Pharmacol. Sin. 22, 1094–1098.