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EXPERIMENTAL STUDY

Trehalose and carnosic acid induced LC3I, LC3II ratio, P62 down-regulation and cleaved caspase 3 expression in neural stem cells

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ABSTRACT

BACKGROUND: Using neural stem cells (NSCs) in cell therapy and regenerative medicine is a growing knowledge. In this study, the protective role of carnosic acid and trehalose against H_2O_2-induced oxidative stress in autophagy induction and apoptosis inhibition in NSCs was investigated.

MATERIAL AND METHODS: The bone marrow stromal cells (BMSCs) were isolated from the femur of the rat and differentiated into NSCs using basic fibroblast and epidermal growth factors (bFGF and EGF), and B27 serum free media. To evaluate the autophagy, the P62 protein was assessed by immunocytochemistry and LC3II / LC3I ratio by Western blotting. Further, we used 3-Methyladenine (3-MA), a widely used autophagy inhibitor to study whether combined treatment of 3-MA with carnosic acid and trehalose modulates autophagy in NSCs. For studying apoptosis, the cleaved caspase-3 protein was evaluated. Carnosic acid and trehalose increased the survival of the NSCs.

RESULTS: The H_2O_2 decreased the autophagy and induced apoptosis with increasing time during 24 hours, however, a pre-treatment with 2 μM carnosic acid and trehalose 3 % induced the autophagy proteins (while increasing the LC3II / LC3I ratio and decreasing the P62) and decreased the apoptosis (while decreasing the expression of the cleaved caspase-3). The results showed that the carnosic acid and trehalose increased the survival of NSCs against the oxidative stress caused by H_2O_2, decreased apoptosis, and induced autophagy.

CONCLUSION: Due to the carnosic acid and trehalose unique properties and its low toxicity, it can be used as an agent in cellular transplantation for reducing oxidative stress and inducing autophagy *(Fig. 4, Ref. 37)*.

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KEY WORDS: carnosic acid, trehalose, neural stem cells, LC3I, LC3II, P62.

Introduction

The adult stem cells such as neural stem cells (NSCs) play an important role in cell and gene therapy approaches in combination with drug usage in the treatment of neurological diseases (1). Due to the availability and abundance of BMSCs, the autologous transplantation capability and non-rejection properties of the transplant, they have been widely considered in the restorative medicine. Due to the low capacity of the NSCs in the central nervous system to replenish damaged cells, other alternative methods have been considered. One of the approaches is cell therapy using other available sources of stem cells, such as BMSCs, and then their differentiation into NSCs. However, these cells quickly disappear after being transplanted into the affected area due to the oxidative stress caused by the reactive oxygen species (ROS) and reactive nitrogen species (RNS) or hypoxic conditions, as well as being deprived of serum. Therefore, the ideas of prolonging the survival of these cells, play an important role in increasing their efficacy in cell transplantations. The NSCs suffer from apoptosis and cell death due to the oxidative stress, hypoxia and inflammatory factors in the affected area after transplantation. In this regard, autophagy plays a key role against the oxidative stress in controlling cellular homeostasis. The oxidative stress leads to the accumulation of destroyed proteins in a cell and the onset of autophagy or apoptosis in the cell. P62/SQSTM1 is a polyubiquitin-binding protein in the inclusion bodies of cytosol along with polyubiqui-

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tinated proteins in the neurodegenerative diseases and cytotoxicity states. The P62 also binds to LC3 and the ubiquitin protein, guiding the ubiquitin complex to the autophagosome for destruction. The LC3II is a substrate for the onset of autophagy and the formation of autophagosomes. In some studies, the formation of the LC3 is proposed as an indicator of autophagy. The conversion of LC3I to LC3II represents autophagy, and higher LC3II levels indicate an increase in the formation of autophagy vacuoles in the cell (2). In the case of apoptosis, the caspases (cysteinyl aspartate-specific proteases), a family of signaling molecules activate and cause degradation of proteinous components of the cell. The Caspase-3 protease, as an "effector" caspase, is the initiator of the apoptosis. Therefore, it is known as an apoptotic marker. It is activated by caspase-8 and caspase-9 (3). Carnosic acid (Salvin) which is present in the rosemary plant (*Rosmarinus officinalis*) has antioxidant, antimicrobial, anticancer, anti-apoptotic and anti-inflammatory properties (4). Previous studies have shown that the carnosic acid prevents oxidative stress-induced cellular damage through activating the main protein of the antioxidant (nuclear factor erythroid 2-related factor 2 (Nrf2)), leading to the inhibition of the cellular damage (5). Trehalose is a non-reducing disaccharide with two glucose molecules present in plants, fungi, and insects (6) and produced in yeast, in response to environmental stressors (7). The neuroprotective role of trehalose has been demonstrated in many studies (8, 9). Trehalose directly prevents protein aggregation, stabilizes proteins, and prevents protein misfolding (10). This sugar, due to its physiological and chemical properties, has received a significant attention by many researchers (11). This sugar enters into cells through endocytosis and pinocytosis and has anti-inflammatory and anti-aging properties (10, 12). In this study, the protective effects of carnosic acid and trehalose in autophagy induction in NSCs have been evaluated.

Materials and methods

Animals

In this experimental study four adult female Wistar rats, aged 6–8 weeks, purchased from the Iranian Pasteur Institute were used. They were kept in a 12-hour light and dark period at animal lab located at the Qazvin University of Medical Sciences. The study was conducted in accordance with the guidelines for working with experimental animals set by the ethics committee of Qazvin University of Medical Sciences (ethic code: IR.QUMS.REC.1397.190).

Cell culture

Rats were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) i.p. A small hole (2 × 3 mm) in the femoral bone was made with an air drill following skin incision (1 cm), and 0.5 mL of bone marrow was aspirated with a 21-gauge needle. BMSCs were isolated from the lower limb bones and washed three times with sterile saline buffer phosphate (PBS) and rats were then sacrificed. The aspirate was taken to tissue culture flasks and the adherent BMSC's were culture expanded until the third passage in culture medium of Dulbecco modified Eagle's medium: F12 (DMEM/F12) along with 10 % FBS (fetal bovine serum), 100 µg/ml penicillin, 100 U / mL streptomycin, and in a 5 % CO_2 incubator, 95 % humidity and a at a temperature of 37 °C.

Differentiation of BMSCs into NSCs

After trypsinization of the BMSCs at the third passage, 10000 cells were seeded equally into each well of the 12-well plates (with one coverslip in each well). In this stage, the BMSCs were cultured in the DMEM/F12 medium (GIBCO-BRL, Germany) containing 2 % of B27 (Invitrogen, Scotland) and 20 ng ml^{-1} of basic fibroblast growth factor (bFGF, Chemicon, Germany) and 20 ng ml^{-1} of epidermal growth factor (EGF, Sigma, Steinheim). After one week, neurospheres with spherical structure were collected by centrifugation. After removing the culture medium, by trypsinization and mechanical digestion, the cells of the neurospheres became single and were re-seeded to form neuroepithelial-like cells and incubated to induce BMSCs-derived-NSCs. Immunocytochemistry technique was used to evaluate the expression of the Nestin protein (the marker of NSCs).

Immunocytochemistry

To confirm the mesenchymal origin of the BMSCs in the third passage and their differentiation into the NSCs, the cells were assessed in two stages, before and after differentiation into the NSCs. To investigate the mesenchymal origin of the isolated BMSCs in the third passage, after cell detaching using trypsin, 5000 of the cells were transferred into each of the spaces of the 6-part lamellated-plates. The stages of the immunocytochemistry testing were conducted similarly to the recommended protocols. In brief, the cells were placed in a 4 % paraformaldehyde solution for 20 minutes. After washing with phosphate buffer, the cells were placed in the 0.3 % Triton X-100 for 15 minutes. After rewashing the cells with phosphate buffer, they were exposed to the antibody at 4 °C for 24 hours. The primary antibodies included CD31 (endothelial cell markers), CD90 (indicative of mesenchymal stem cells), CD34 (indicative of hematopoietic stem cells) and Nestin (a NSCs marker), all from the ABCAM Company. The cells were washed with PBS and the fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1 : 100; Chemicon), seen as green, was added to the cells for 2 hours at room temperature. Propidium iodide (PI) was used for the counter staining. The cells with positive immune response were counted by a fluorescence microscope (13).

Viability test

Prior the experiments, the viability tests via trypan blue were conducted on the cells to determine the H_2O_2 toxicity and the protective effect of carnosic acid. A number of 1000 of cells were distributed into a 96-well microtiter. To determine the H_2O_2 toxicity, the survival of the cells at different doses of H_2O_2 (0, 50, 100, 200 and 400 µM) was estimated for 24 hours. Moreover, in order to evaluate the protective effect of carnosic acid and trehalose, the cells were pretreated with 2 µM carnosic acid and trehalose two hours before being exposed to H_2O_2 (carnosic acid + H_2O_2 group). To monitor the survival of the cells, an equal volume of cell suspension and the same volume of trypan blue mixture were used, and the counting was performed using a Neobar lamella under

the microscope. In this method, the dye penetrates the dead cells, makes them blue, and the non-dyed cells represent alive cells. Then, through counting all the cells including the dyed ones, the percentage of the live cells can be obtained. For each cell group, counting was performed three times under a microscope. The protective role of carnosic acid against the damage induced by ROS produced by H_2O_2 was evaluated by studying the apoptosis activity of cleaved caspase-3 and autophagy (LC3, P62).

Western blot analysis

After treatment of the cell groups as described, the proteins of the cells were extracted and frozen at –80 °C. The proteins were separated in a 15 % SDS-PAGE gel and transferred to the polyvinylidene fluoride (PVDF) membrane. The membrane was blocked by 5 % fat-free powdered milk for 30 min, then, washed three times with TBST and subjected to cleaved caspase-3 (Asp175), LC3, P62 and GAPDH antibodies at 4 °C for 24 hours. The membrane was washed three times with TBST and incubated with a secondary antibody of goat anti-mouse or rabbit IgG peroxidase (1 : 10000–1: 20000) for 1 hour. The membrane was washed three times by TBST, and the image was formed in the membrane by an enhanced chemiluminescence kit (ECL) and a densitometric analysis was performed by the Image J software (GE Healthcare Life Sciences). To determine the effect of autophagy on NSCs, we used 3-MA (3-methyladenine, Sigma-Aldrich) as an autophagy inhibitor. 3-MA was dissolved in phosphate buffer saline (PBS, Invitrogen) at 1 Mm concentration. We measured the autophagic activity of carnosic acid – trehalose treated NSCs with or without pretreatment with 3-MA by western blot.

NSCs transfection by pEGFP-LC3 using lipofectamine 2000

In order to study the autophagosome formation, the NSCs were temporarily transfected with pEGFP-LC3 by lipofectamine 2000 (Invitrogen). In short, the cells were cultured in a petri dish and after the attachment of the cells to the bottom of the dish; the culture medium was replaced by the Opti-MEM reduced-serum. The complex containing DNA (0.4 mg of DNA and 5 ml of lipofectamine) was added to the cells and incubated for 4 hours. Then, the cells were washed and placed in a culture medium without antibiotics for 24 hours. After that, the cells were placed in the H_2O_2 medium, as described earlier. The vesicles formed by GFP-LC3 indicate the number of autophagosomes formed, which were examined using a confocal microscope.

Results

BMSCs isolation and differentiation into NSCs

After being isolated, BMSCs look spherical and were stuck to the bottom of the flask. After 48 hours and following washing with PBS, the floating cells that are not attached to the bottom of the flask were washed. After the third passage, the BMSCs were evaluated in terms of stemness and mesenchymal progeny by means of the immunocytochemical study of surface markers. The BMSCs showed a negative reaction for the CD31 antibodies (endothelial stem cells marker) and CD34 (hematopoietic stem cells marker), but the cells showed a positive immune reaction to the CD90 antibody (mesenchymal stem cells marker) and CD106 (bone marrow stem cells marker) (Fig. 1). BMSCs were immunolabeled with primary antibody, incubated with FITC-conjugated secondary antibody (green) and counter-stained using propium iodine (red). After differentiation of the BMSCs to the NSCs, the expression level of Nestin protein as a marker for NSCs showed a significant increase (expression level was 92 %) as compared with the BMSCs (Fig. 1). Moreover, after differentiation, the low expression level of CD90 protein (expression level was 6 %) indicated the differentiation of the BMSCs into NSCs (Fig. 2).

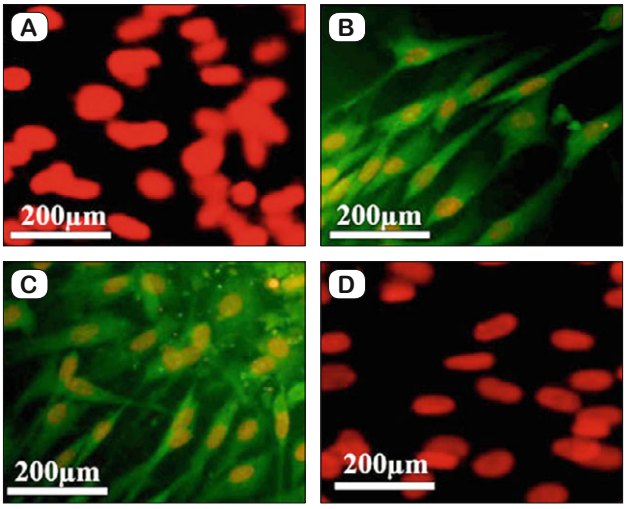

Fig. 1. Characterization of isolated BMSCs. (A) immunostaining of CD31 (endothelial stem cells marker), (B) CD90 (mesenchymal stem cells marker), (C) CD106 (bone marrow stromal stem cells marker) and (D) CD34 (hematopoietic stem cells marker). Cells were immunolabeled with primary antibody, incubated with FITC-conjugated secondary antibody (green) and counter-stained using propium iodine (red).

Viability test results

In the viability test, the NSCs without H_2O_2 were considered as the control group. Four groups of cells were treated with different concentrations of H_2O_2 for 24 hours. The survival rates were 82 %, 72 %, 49 %, 39 % of the control group in the 50, 100, 200, 400 µm H_2O_2 group, respectively (Fig. 2 A). The cell death rate in cell groups which received 200 and 400 µM H_2O_2, for which the survival rates went below 50 %, had a significant difference ($p < 0.05$) from the control group (Fig. 2A). To evaluate the protective effect of carnosic acid and trehalose, NSCs were pretreated 2 hours before exposure to H_2O_2 with 2 mM carnosic acid and trehalose. The results showed that pre-treatment increased the cell survival (Fig. 2B). The cell survival was 94 %, 87 %, 71 %, and 50 % in the medium containing 2 µM carnosic acid- trehalose 3 % and H_2O_2 at concentrations of 50, 100, 200, 400 µM, respectively (Fig. 2B). A comparison of the H_2O_2 treated cells with and without carnosic acid and trehalose has been shown in Figure 2A and B, which revealed that carnosic acid and trehalose significantly increases cell survival and decreases cell apoptosis resulted by H_2O_2.

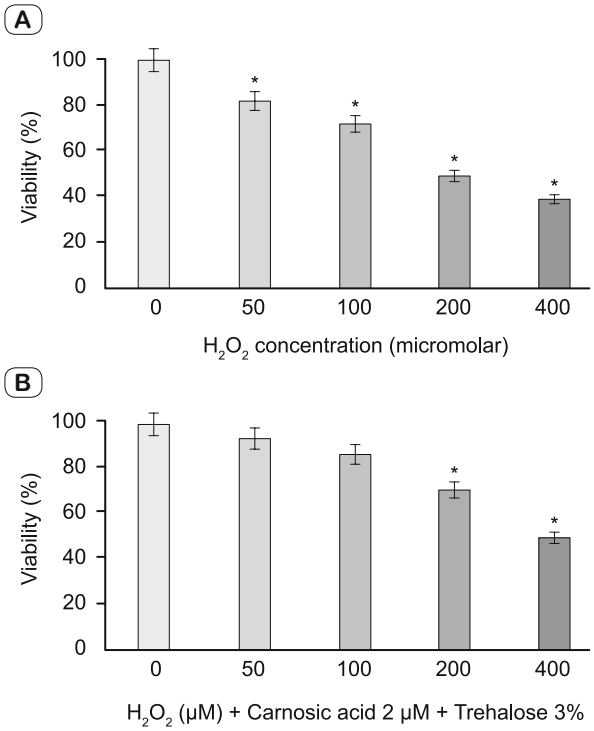

Fig. 2. Cell viability was determined by trypan blue assay. (A) Dose-dependent cell death induced by H_2O_2 in NSCs. (B) Carnosic acid 2 μM and trehalose 3 % pre-treatment protects NSCs against H_2O_2-induced cell death Asterisks indicate significant differences with the control ($p < 0.05$).

Carnosic acid and trehalose induced autophagy

The NSCs with and without carnosic acid and trehalose were exposed to H_2O_2 at 50 and 100 μM concentrations for 24 hours to determine whether H_2O_2-induced oxidative stress activates the autophagy pathway. The level of autophagy was evaluated by determining the ratio of LC3II to LC3I. The ratio of LC3II to LC3I decreased 24 hours after being treated with 100 μM H_2O_2, but in the medium containing 2 mM carnosic acid-trehalose 3 % and H_2O_2 (50 and 100 μM), this ratio increased (Fig. 3A). In addition to LC3 lipidation, the P62 protein is also an autophagic marker. P62 is attached to LC3 and then decomposed by the pathway of autophagy lysosomes. Therefore, a high level of P62 represents a problem in autophagy. In the case of autophony induction, P62 should be low. In this study, it was shown that P62 increased with a rise in the H_2O_2 concentration so that the highest P62 relates to the H_2O_2 without treatment with carnosic acid-trehalose after a passage of 24 hours, indicating an inhibition of autophagy (Fig. 3A). Next, the proteolytic activity of the cleaved caspase 3 was evaluated as an apoptotic marker. 2 μM of carnosic acid-trehalose 3 % reduced the activity of the apoptotic marker of the cleaved caspase 3 against H_2O_2 (Fig. 3A). The presence and absence of Carnosic acid-trehalose decreased and increased the activity of cleaved caspase 3, respectively (Fig. 3A). In a medium containing 50 μM of H_2O_2, 2 mM carnosic acid-trehalose 3 %, inhibited the activation of the cleaved caspase 3. However, with increasing the H_2O_2 dose to 100 μM, carnosic acid-trehalose 3 % lost the ability to inhibit cleaved caspase 3 (Fig. 3A).

To investigate the role of carnosic acid-trehalose induced autophagy of NSCs, we used 3-MA, a classical inhibitor of autophagy, to inhibit autophagy. We measured the autophagic activity of carnosic acid-trehalose treated NSCs with or without pretreatment with 3-MA. Interestingly, 3-MA, a class III PI3K inhibitor that blocks autophagy, attenuated CA-induced autophagy at 1 mM of concentration, while combined carnosic acid-trehalose and 3-MA co-treatment markedly inhibited LC3 conversion and p62 down-regulation (Fig. 3B).

The formation of the puncta containing LC3 is a characteristic of autophagic activity. The NSCs were transiently transfected for 24 hours by a plasmid containing GFP-LC3 (pEGFP-LC3, Addgene, Plasmid #21073), which is seen as green fluorescence emissions. The transfected cells were divided into three groups. First group was not exposed to H_2O_2 as control. The second group consisted of the pre-treated cells with 2 μM of carnosic acid-trehalose 3 % and H_2O_2 at a concentration of 50 μM. The third group was exposed to 50 μM H_2O_2 only. The treatment time was 24 hours, and the cells were evaluated with a fluorescence microscope (Fig. 4). In the control group, the fluorescent green color was distributed uniformly throughout the cytoplasm. In the second group, the treated cells with carnosic acid-trehalose and H_2O_2, the vesicular-like puncta containing GFP-LC3 looked bright green; however, more bright vesicles were seen in the third group. These bright spots indicate the formation of autophagosomes following the oxidative stress induction. After the counting, it was noted that the number of points was much higher in group III comparing other groups (Fig. 4D).

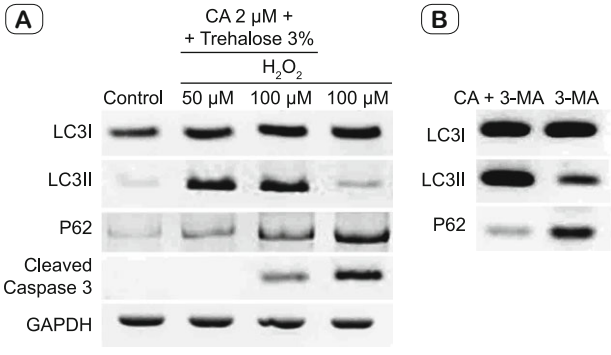

Fig. 3. Western blotting analyses of LC3, P62, and caspase-3 expressions. (A) The NSCs were treated with 2 μM carnosic acid and trehalose 3 % (CA) and exposed to 50 and 100 μM of H_2O_2 for 24 hours. Autophagic flux was examined by means of LC3 II/I conversion using Western blotting with LC3 antibody. LC3I, LC3II, P62 and cleaved caspase 3 were detected using specific antibodies. GAPDH was used as an internal control. (B) NSCs were exposed to 2 μM CA with or without 1mM 3-MA for 24 h. Levels of LC3I, LC3II and P62 were determined by western blotting. CA -induced autophagy is inhibited by 3-MA in NSCs.

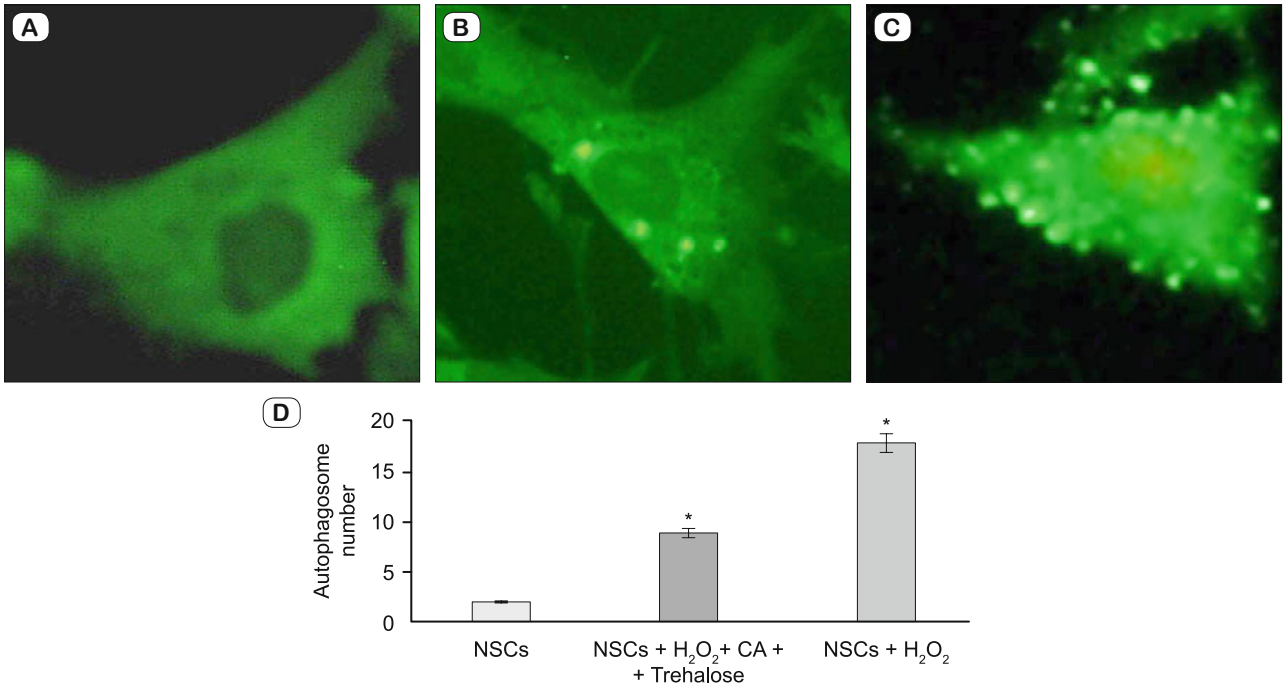

Fig. 4. NSCs were transfected with pEGFP-LC3 plasmid for 24 hours; the cells were then treated with H_2O_2 and carnosic acid (CA) and trehalose in (B) and H_2O_2 alone in (C). Images were taken under the fluorescent microscope. Autophagosome numbers were counted after H_2O_2 exposure in (D); statistical significance among the control group and each treatment group was determined by means of analysis of variance.

Discussion

This study examined the role of carnosic acid-trehalose against the oxidative stress induced by H_2O_2 in the NSCs while considering the relationship between apoptosis and autophagy in cells modulated by carnosic acid-trehalose treatment. It was shown that the pre-treatment with 2 μM of carnosic acid-trehalose 3 % protects the NSCs against H_2O_2 and induces autophagy. However, in a culture medium free of carnosic acid-trehalose, H2O2 causes apoptosis in the NSCs after 24 hours. Carnosic acid-trehalose probably prevents apoptosis induced by oxidative stress through activating autophagy. In the oxidative medium free of carnosic acid-trehalose, the result was the irreversible cell damage, and the ultimate outcome was cell death through apoptosis. The oxidative stress produces ROS, resulting in lipid peroxidation, DNA and RNA damage, inactivation of most cellular enzymes and activation of apoptosis-related enzymes such as caspases (14). The NSCs, after being transplanted, experience apoptosis and the cell death will initiate due to oxidative stress in the cell niche. As previously reported, the oxidative stress induces autophagy in the cell, sometimes leading to apoptosis and cell death (14). Although the mechanism is different between autophagy and apoptosis, there are cellular signaling pathways, which interact with these two phenomena (14). Autophagy is a self-clearing cellular mechanism for removing and rehabilitating the destructed organelles and proteins in the cells. Recent studies have shown that autophagy is activated against environmental stresses such as hypoxia and starvation (15). Also, it has been found that a problem in the initiation of autophagy, elongation, and complementation causes illnesses such as cardiovascular diseases (16), aging (17), cancers (18) and neurodegenerative diseases (19). A study revealed that carnosic acid prevents H_2O_2-induced mitochondrial damage leading to apoptosis in SH-SY5Y cells (20). In other studies, carnosic acid has been shown to protect cells against a variety of oxidative stresses applied to the cells (5). It is possible that carnosic acid acts in multiple pathways as it has been previously confirmed that carnosic acid activates the cellular anti-oxidant system through activating Nrf2 (21). In a recent study, researchers observed that carnosic acid, in a concentration as low as one micromol, protects hepatocytes from cell death caused by H_2O_2 cytotoxicity through activating Nrf2, but a 10 μM concentration of carnosic acid augmented apoptosis (22). In another study, carnosic acid protected the SH-SY5Y cells from the H_2O_2-induced oxidative stress through activating Nrf2 (23). It was also found that carnosic acid prevents HepG2 cells from apoptosis by activating the signaling pathway of Sirtuin1 (22). Carnosic acid, in another study, saved the SH-SY5Y cells from apoptosis induced by amyloid-β toxicity through an induction of autophagy (24). The protective role of carnosic acid in SH-SY5Y cells has been shown through Erk1/2 and Akt phosphorylation and FoxO3a dephosphorylation and autophagic induction (25). One micromole of carnosic acid protected the SH-SY5Y cells for 12 hours by activating the PI3K/Akt and Nrf2 pathway against oxidative stress, and attenuated cellular apoptosis resulting from the toxicity of paraquat (26).

Many studies have shown that carnosic acid is a strong activator of Nrf2, which forms a complex with Kelch-like ECH-associated protein (KEAP1). Then, Nrf2 is transferred to the cell nucleus, leading to the expression of antioxidant enzymes (26, 27). Interestingly, P62 protein sequesters KEAP1 into the autophagosomes impairing Nrf2 ubiquitylation, leading to of Nrf2 signaling pathway activation (28, 29). It has been shown that KEAP1-P62-LC3 protein complex formation is necessary for ubiquitin aggregates clearance in response to oxidative stress (30). In a study, within a carnosic acid-free medium or a concentration of 100 μM H_2O_2, the cells expressed the cleaved caspase 3 after 24 hours, and a decreased ratio of LC3II/LC3I, which is an autophagic index. In a medium containing H_2O_2 at a concentration of greater than 100 μM, the carnosic acid is no longer able to protect the cells and the autophagy was directed towards apoptosis. The cellular death phase starts with autophagy inhibitors (activating executioner caspases, caspase-3/7 and caspase-6) (31). Many studies have shown the role of autophagy and apoptosis in the stressful conditions and cellular signals leading to apoptosis in NSCs. Numerous studies have revealed the varying sensitivity of cells to H_2O_2. For example, retinal pigmented epithelial cells are resistant to H_2O_2 at concentrations more than 400 μM even up to 24 hours (32) but neurons are highly susceptible to H_2O_2-induced oxidative stress and a concentration of 5–10 μM of H_2O_2 leads to neuron toxicity so that autophagy leads to cell death rather than playing the protective roles (33). Studying is ongoing in many aspects to find out new pharmaceutical approaches for protecting cells and tissues.

The protective role of trehalose by induction of autophagy has been shown in many studies (8, 9, 34). In one study Trehalose has neuroprotective effects on the substantia nigra dopaminergic cells by activating autophagy and non-canonical nrf2 pathways (9). Saccharides play essential roles in brain development, synaptogenesis, synaptic transmission, and neurotransmitter production (35). Researchers show Trehalose alleviates oxidative stress-mediated liver injury via activating autophagy in mice (36). In this study, 3 % trehalose and carnosic acid increased the cellular survival and the rise in the autophagy activity. It is likely to prevent cell death by increasing autophagy and eliminating the organs and proteins damaged by H_2O_2. In this study, H_2O_2 with a concentration more than 100 μM increased cell death. In this study, in the carnosic acid and trehalose-free environment and H_2O_2, the cells expressed the cleaved caspase 3 protein, and the ratio of LC3II/LC3I, which is the autophagic index, decreased. The activation of caspases plays a major role in the cell death through apoptosis (37).

Conclusion

Carnosic acid and trehalose increases autophagy and decreases apoptosis in bone marrow derived stem cells. It regulates the cellular defense system against cell death through autophagy activation.

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