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Research Paper:

Protective Effect of Trehalose Against H_2O_2-induced Cytotoxicity and Oxidative Stress in PC-12 Cell Line and the Role of Heat Shock Protein-27

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ABSTRACT

Background: Oxidative stress has been shown to be an important factor, which plays a significant role in the pathogenesis of neurodegenerative disorders. Heat Shock Protein-27 (HSP-27) has been implicated in antioxidant responses against oxidative stress. Trehalose is a natural disaccharide widely used in a variety of food products with demonstrated protective effects against several neurodegenerative diseases. This study investigated the effects of trehalose on antioxidant responses, and the gene expressions for HSP-27 and caspase-3 against hydrogen peroxide (H_2O_2) induced oxidative injury in PC-12 cell line.

Methods: The PC-12 cells were treated with various concentrations of H_2O_2 and trehalose for 24hr. The cell viability was assessed, using MTT and Lactate Dehydrogenase (LDH) release assays. Moreover, the activity of Catalase (CAT) and Glutathione Peroxidase (GPx) enzymes, and the Malondialdehyde (MDA) levels were determined. In addition, the levels of HSP-27 and caspase-3 gene expressions were measured.

Results: The results indicated that the pretreatment with trehalose increased cell survival against the H_2O_2-induced oxidative injury. Furthermore, trehalose elevated the CAT and GPx activities and reduced MDA levels compared to that of control group ($P<0.05$). Moreover, trehalose upregulated the HSP-27 gene expression, while reducing the expression of caspase-3 gene compared to that of the untreated cells ($P<0.05$). All of these biochemical changes were found to be dose-dependent for trehalose.

Conclusion: Based on the study findings, trehalose had the capacity to attenuate the oxidative stress and cell injury. Therefore, trehalose may be suggested as a therapeutic agent to treat neurodegenerative disorders caused by oxidative stress damages.

Keywords: Antioxidant, Cell viability, HSP27, Oxidative injury, Trehalose

Introduction

Oxidative stress is a condition developed by an imbalance between oxidant and antioxidant concentrations in biological systems. The imbalance occurs due to excessive production of Reactive Oxygen Species (ROS) and insufficient activity of antioxidant defense system. It is well documented that oxidative stress is implicated in the pathogenesis of neurodegenerative conditions, such as Alzheimer's and Parkinson's diseases [1, 2]. Indeed, extensive levels of ROS cause lipid peroxidation, protein misfolding and aggregation, together with DNA damages, which ultimately result in neuronal apoptosis via both intrinsic and extrinsic pathways [3]. Hydrogen peroxide (H_2O_2), one of the main reactive oxygen species, is normally produced in various redox processes and is considered as a messenger in intracellular signaling pathways [4].

The rise in H_2O_2 during oxidative stress is; however, believed to induce DNA damages and cell apoptosis [5]. The induced apoptosis has been associated with alterations in apoptotic and anti-apoptotic proteins. Caspase-3, a cysteine protease known to be a key executor involved in preprogrammed cell death, is activated by H_2O_2 as a final effector in the apoptosis process [6]. In addition, H_2O_2 mediated oxidative stress induces Heat Shock Protein (HSP-27), which is a member of this superfamily [7, 8]. Heat shock proteins are a group of ubiquitous and conserved proteins, providing an intrinsic mechanism to defend cells against various physiological stresses [9]. The HSP-27 belongs to the small molecular weight proteins, which exerts its cytoprotective property through inhibiting oxidative stress and apoptosis.

The overexpression of HSP-27 has been shown to prevent apoptosis by inhibiting caspase activation in various cellular stresses, including accumulation of misfolded proteins, generation of ROS and DNA damages [9-11]. Therefore, developing pharmacological interventions to induce HSP-27 might be a promising strategy in the treatment of neurodegenerative diseases in humans. Trehalose is a naturally occurring, nonreducing disaccharide, consisting of two glucose moieties [12]. It is widely present in various organisms except for vertebrates [13]. Trehalose is involved in adaptive responses to osmotic stress, extreme temperature and anhydrosis *in vivo*. It also stabilizes native proteins and protects membrane integrity during various biological stresses [14]. Trehalose is widely used in food products because of its unique stabilizing, texturizing and sweetening properties [15]. The cytoprotective effect of trehalose against oxidative stress has been reported by previous studies [16, 17]. We have recently demonstrated that trehalose protects the enzyme chondroitinase-ABC against oxidative stress and proteolysis [18]. We have also reported that trehalose treatment reduces inflammatory responses and oxidative stress induced by traumatic spinal cord injury [19]. However, whether trehalose exerts its protective action through modulating HSP27 remains unclear.

This study aimed to investigate the underlying protective mechanism of trehalose in H_2O_2-treated PC12 cells.

Materials and Methods

Cell culture & treatment: Rat Pheochromocytoma cells (PC-12) were obtained from Pasteur Institute (Tehran, Iran) and cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) heat-inactivated fetal bovine serum and a 1% antibiotic mixture, consisting of penicillin and streptomycin under humid condition of 5% CO_2 at 37°C. The culture medium was changed every second day. The cells were pretreated with trehalose at 0, 12.5, 25 or 50 mM, incubated for 24hr, and were subsequently subjected to freshly prepared H_2O_2 at a final concentration of 100µM, and incubated for another 24hr.

MTT assay: The cell viability was determined by the conventional MTT reduction assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The PC-12 cells were seeded in 96-well plates at a density of 5×10^3 cells per well, and incubated for 24hr before the subsequent experimental steps. The cells were then subjected to the specific treatments of interest per group. After incubation for 24hr, The MTT reagent was added to each well (0.5 mg/ml) and the plates were incubated at 37°C for 2hr. The intracellular formazan product was then dissolved in 200µl of DMSO. The absorbance of the solution in each well was then read at 490nm, using a microplate spectrophotometer, and the results were expressed as the percentages compared to the control group.

Lactate Dehydrogenase (LDH) assay: The cell death was assessed by measuring the LDH release in the culture medium. The PC-12 cells were cultured with varying concentrations of trehalose (0, 12.5, 25 or 50 mM) and then treated with H_2O_2 at 100µM, as described previously. The H_2O_2 concentrations were determined based on the results of MTT assay. The cell culture solutions were centrifuged at 1500×g for 5 min and the supernatants were collected. For the LDH activity assay, the reduction in the NADH absorbance at 340 nm was measured in phosphate buffer in the presence of pyruvic acid as the LDH substrate [7]. The LDH release was expressed as the percentages of the values recorded for the controls.

Catalase, glutathione peroxidase & malondialdehyde assays: To investigate the activity of Catalase (CAT) and Glutathione Peroxidase (GPx) and the Malondialdehyde (MDA) level, the PC-12 cells were cultured in 6-well plates. After the treatment as described earlier, cells were detached, collected and washed in cold Phosphate-Buffered Saline (PBS) and homogenized. The homogenates were subsequently centrifuged at 12000g for 10 min at 4°C. The supernatants were collected and stored at -70°C for the subsequent assays. The CAT and GPx activities were determined, using commercial kits, based on the manufacturer's assay protocols. The MDA content was also assayed, using thiobarbituric acid method [20]. The protein concentration in each sample was measured by Bradford method, using bovine serum albumin as the standard [21].

Isolation of RNA and real-time PCR: The total cellular RNA for each cell sample was isolated, using trizol reagent. The concentration of total RNA was measured for each sample on a Nano-Drop ND-1000 spectrophotometer (Thermo Scientific, USA). Then, 0.5µg of total RNA was reverse transcribed to cDNA, using a cDNA synthesis kit. The specific gene primers for HSP-27, caspase-3 and Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) were designed by Oligo-Analyzer software (Table 1). The selected genes were amplified, using cDNA, SYBR Green master mix, and both forward and reverse primers. The PCR conditions were as follows: initial denaturation for 10 min at 95°C → 40 cycles of denaturation for 40 sec at 95°C → annealing for 25 sec at 66°C, and → extension for 15 sec at 72°C. The mRNA levels were expressed relative to GAPDH mRNA in the same sample, using $2^{-\Delta\Delta CT}$ method [22].

Statistical analyses: All experiments were performed in triplicates at minimum. The statistical differences among groups were analyzed, using one-way Analysis of Variance (ANOVA) followed by Tukey's post hoc test on SPSS software, version 20 (IBM, USA). The data were expressed as the Mean±SD for the experimental and control groups. The statistical differences were considered as being significant at $P<0.05$.

Results

Effect of trehalose on PC-12 cells viability: The effect of trehalose on PC-12 cell viability was studied at varying concentrations of trehalose. The results demonstrated that the cell viability declined to 90% in the presence of trehalose at the concentrations of 12.5, 25 and 50 mM, respectively. However, no significant differences were observed among these treatments ($P\leq0.05$) (Figure 1A), hence these concentrations were used in the subsequent experimental steps. Investigating the viability of cells exposed to H_2O_2 showed a dose-dependent reduction in cell survival. At 100µM H_2O_2, the cell viability decreased to 46.8% compared to that of the control group (Figure 1B). Therefore, this concentration was used for the subsequent experiments. As shown in Figure 1C, pretreatment of cells with trehalose at 12.5, 25 or 50 mM prevented H_2O_2–induced cell death and improved the cell viability to 56.5%, 62.2% and 70%, respectively.

Effect of trehalose on LDH release: The protective effect of trehalose was further investigated by LDH release assay. The results showed that the LDH release increased significantly in the H_2O_2–treated group compared to that of the controls ($P<0.01$). In contrast, the pretreatment with trehalose at 12.5, 25 or 50 mM reduced the LDH release dose-dependently (Figure 1D).

Effect of trehalose on the CAT, GPx and MDA levels: The ability of trehalose to protect against the oxidative stress induced by H_2O_2 was determined by measuring the CAT and GPx activities, and the MDA level in each cell group. The results indicated that following exposure of cells to H_2O_2, the CAT and GPx activities decreased significantly compared to those of the control group ($P<0.05$ and $P<0.01$, respectively). However, the pretreatment with trehalose significantly elevated the CAT and GPx activities in a dose dependent manner compared to those of the untreated group ($P<0.01$) (Figures 2A and 2B). Further, the MDA level significantly increased in the H_2O_2-treated cells compared to that of the control group ($P<0.01$). Conversely, the trehalose pretreatment lowered the MDA production in PC-12 cells compared to that of the untreated group ($P<0.05$) (Figure 2C).

Effect of trehalose on the expression of HSP-27 and caspase-3: To determine the neuroprotective effect of HSP-27 in the trehalose-treated group, the mRNA level for the HSP-27 was determined. As shown in Figure 3A, the HSP-27 gene expression was upregulated in H_2O_2–treated cells compared to that of the control group ($P<0.05$). Moreover, pretreatment of cells with trehalose at 12.5, 25 or 50 mM increased the mRNA level for HSP-27 significantly compared to that of the untreated cell group ($P<0.05$ for 12.5 and 25 mM and $P<0.01$ for 50 mM trehalose). Furthermore, H_2O_2 elevated the expression of caspase-3 gene, a key regulator of apoptotic response, compared to that of the control group ($P<0.01$). However, the trehalose pretreatment downregulated caspase-3 gene expression in a dose-dependent manner compared to that of the untreated cell group ($P<0.05$ for 12.5 mM and $P<0.01$ for 25 and 50 mM) (Figure 3B).

Discussion

Previous studies have emphasized that oxidative stress plays a crucial role in the pathophysiology of neurodegenerative diseases. Chemicals, such as hydrogen per-

Table 1. Nucleotide sequencing of the primers used for real-time PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size (bp)
HSP 27	GAAATACACGCTCCCTCCAG	CTGATTGTGTGACTGCTTTGG	110
CASP3	GCTGGACTGCGGTATTGAG	TAGTAACCGGGTGCGGTAG	111
GAPDH	AACCCATCACCATCTTCCAG	GTGGTTCACACCCATCACAA	197

oxide, superoxide anion and hydroxyl radicals damage biomolecules, leading ultimately to cell death by apoptosis [23, 24]. Therefore, removal or suppression of ROS is likely to be an effective strategy to prevent oxidative cell damages or cell death. Hydrogen peroxide is one of the most abundant and stable members of ROS, which has been frequently used to induce oxidative stress [25]. Moreover, rat pheochromocytoma PC-12 cell line is commonly used to study the neuronal damage induced by oxidative stress *in vitro* [26]. Thus, we investigated the cytotoxic effect of H_2O_2 and the protective impact of trehalose on PC-12 cell line. Our results demonstrated that trehalose effectively prevented cell death, and significantly reduced LDH release and the resultant oxidative stress. We also demonstrated that the neuroprotective effect of trehalose was mediated by HSP-27.

Lactate dehydrogenase is a stable cytoplasmic enzyme found in all cells, which leaks to the extracellular space due to cell membrane damages. Therefore, the LDH level in the culture medium is a marker of cellular integrity or damage [27]. In the current study, we evaluated the toxicity of H_2O_2 and the protective effect of trehalose by LDH assay, which correlated with the data from the

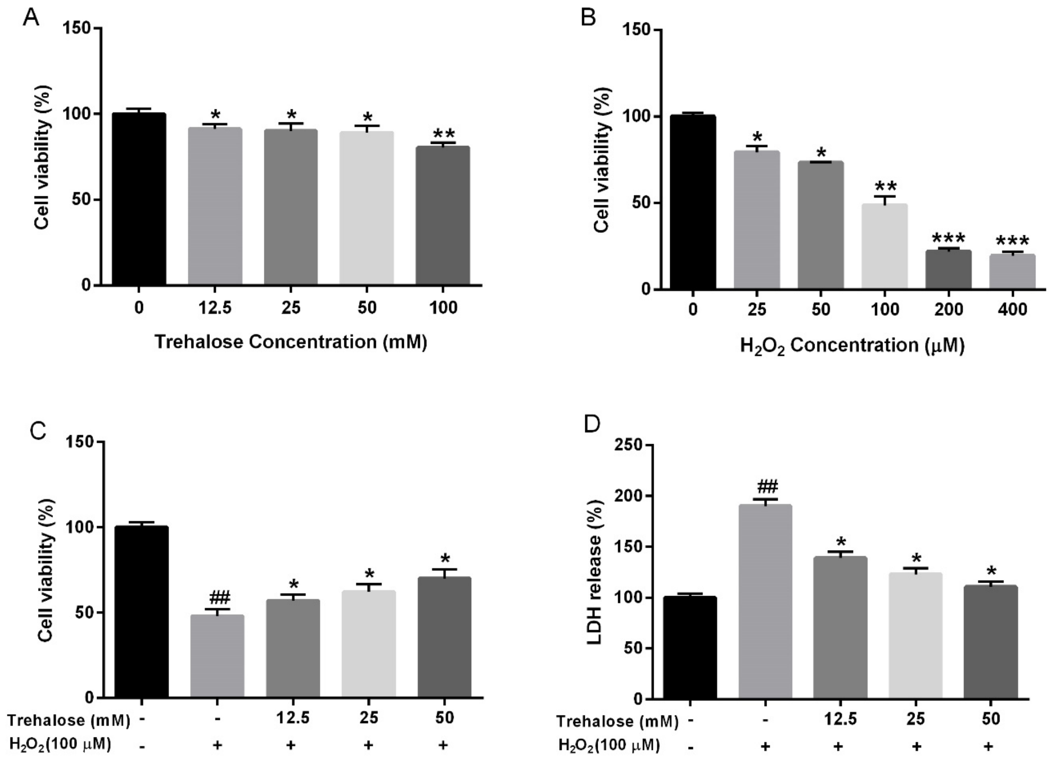

Figure 1. (A) Effect of varying concentrations of trehalose (0-100 mM) on PC12 cell viability

(B) Effect of varying concentrations of H_2O_2 (0-400 µM) on PC12 cell viability.

(C) Effect of varying concentrations of trehalose (0-50 mM) on H_2O_2-induced cytotoxicity.

(D) Effect of varying concentrations of trehalose (0-50 mM) on H_2O_2-induced LDH release.

Data are presented as the Mean±SD of three independent experiments. ##P<0.01 compared to control group; *P<0.05; **P<0.01; ***P<0.001 compared to H_2O_2-treated group.

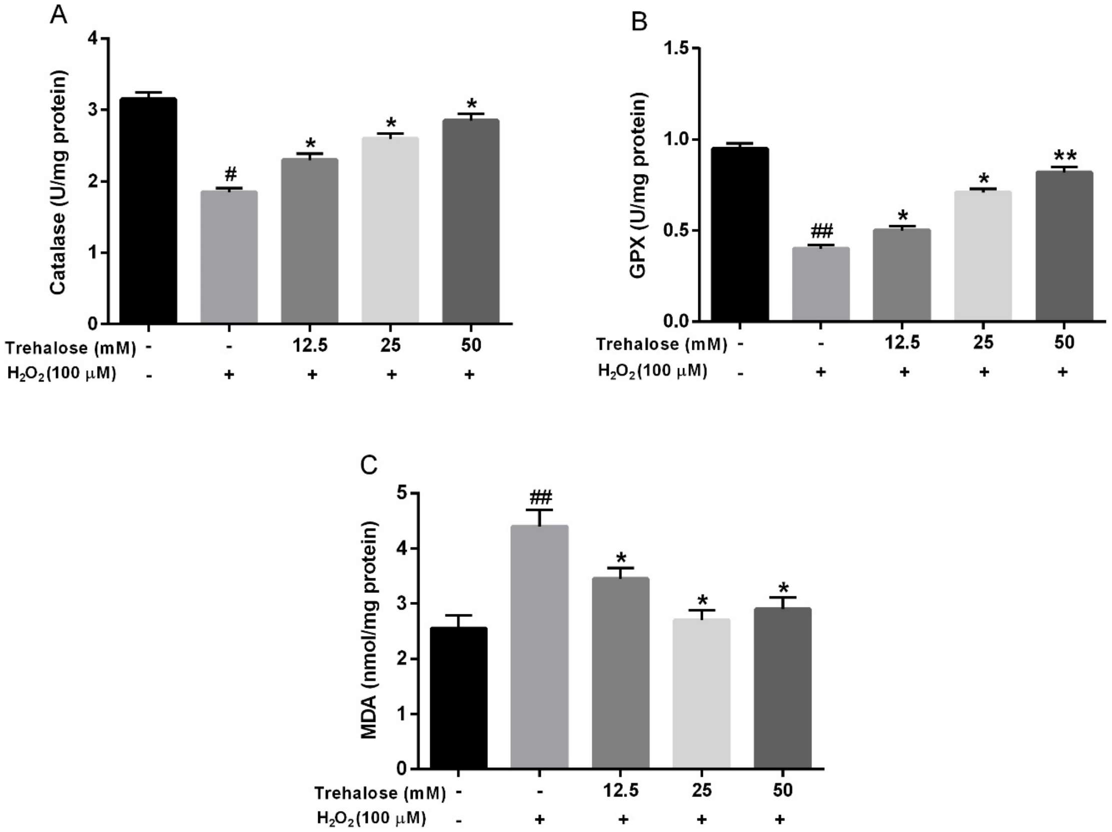

Figure 2. Effect of trehalose pretreatment on the activity of (A) Catalase (CAT) (B) Glutathione Peroxidase (GPx) and (C) the level of Malondialdehyde (MDA) in PC12 cells treated with H_2O_2

Data are presented as the Mean±SD of three independent experiments. #P<0.05; ##P<0.01 compared to control group; *P<0.05; **P<0.01 compared to H_2O_2-treated group.

MTT assay. Our results clearly demonstrated that trehalose rescued H_2O_2–induced neurotoxicity, suggesting that it has neuroprotective effect.

Cells normally contain endogenous antioxidants, which scavenge ROS to prevent cell damages. In this study, H_2O_2 treatment was found to reduce the activity of CAT and GPx in the cells. It also increased the MDA lev-

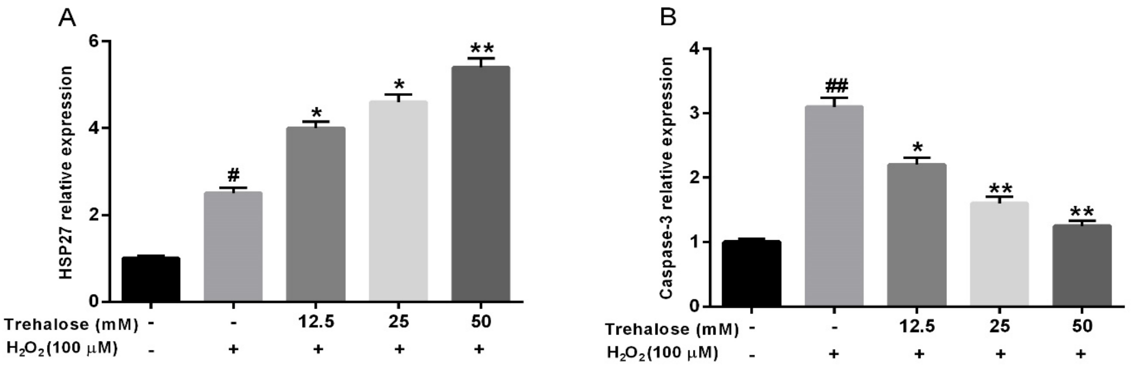

Figure 3. Effect of trehalose pretreatment on relative expression of (A) heat shock protein 27 (HSP27) and (B) caspase-3 genes in PC12 cells treated with H_2O_2

Data are presented as the Mean±SD of three independent experiments. #P<0.05; ##P<0.01 compared to control group; *P<0.05; **P<0.01 compared to H_2O_2-treated group.

els as the marker of lipid peroxidation. The results were in agreement with those reported by previous studies [28, 29]. However, treatment with trehalose significantly increased the activity of these antioxidant enzymes and decreased the MDA levels. These findings were consistent with those achieved from MTT and LDH assays, suggesting the potential capacity of trehalose to protect cells from oxidative damages. In line with these findings, a recent study has demonstrated that trehalose treatment attenuates the oxidative stress induced by cadmium in rat kidney cells [30]. Similar results have also been observed in rat brain tissue [31].

Our results demonstrated that the mRNA gene level for HSP-27 was significantly upregulated in the H_2O_2–treated cells compared to those of the controls. This heat shock protein belongs to the small molecular weight protein family, which responds to cellular stresses, such as heat shock, and oxidative, physical and chemical stressors [11]. Under oxidative stress condition, HSP-27 functions as an antioxidant, scavenging ROS by raising the intracellular levels of antioxidants [32]. Therefore, it is likely that the expression of HSP-27 primarily increased as a protective response to H_2O_2–induced oxidative stress in a dose-dependent manner. Studies investigating the effect of trehalose on HSPs expression are limited, thus supporting the significance of the current study.

We recently reported that trehalose increased HSP-27 and HSP-70 expression in spinal cord tissues following traumatic damages in animal models [19, 22]. We have also shown that trehalose attenuates oxidative response after spinal cord injury by inducing these HSPs. Similarly, the upregulation of HSP-27 in trehalose-treated PC-12 cells caused an increase in the activity of CAT and GPx enzymes, leading to reductions in the MDA levels. However, this phenomenon was not observed in H_2O_2–treated cells which may contribute to the inactivation of CAT and GPx due to overgeneration of ROS. Prolonged oxidative stress can cause cell death through activation of apoptosis [32, 33]. However, HSP-27 prevents programmed cell death particularly by inhibition of caspase-dependent apoptosis.

Therefore, induction of HSP-27 protects cells against oxidative injury and apoptotic cell death. Our findings revealed that caspase-3 expression was upregulated in PC-12 cell line following treatment with H_2O_2. Conversely, the pretreatment with trehalose reversed this trend in a dose-dependent manner. These results are in agreement with our findings achieved from LDH release and MTT assays, suggesting that trehalose improves cell survival under oxidative stress condition through the induction of HSP-27. In line with these findings, a previous study has shown that the damages resulting from the exposure of cultured astrocytes to H_2O_2 were reduced by the induction of HSP-27 and HSP-72, which lowered astrocyte apoptotic cell death from H_2O_2–induced oxidative damages [7].

Conclusions

This study demonstrated that H_2O_2 induces oxidative damages in PC-12 cells. The findings also revealed that the pretreatment with trehalose protects these cells from H_2O_2-induced oxidative stress as indicated by improvement observed in the cell viability and reduction in the LDH release. Trehalose increased the activity of antioxidant enzymes and reduced the extent of lipid peroxidation, thereby restored antioxidant defense and attenuated the ROS generation. The neuroprotective effects of trehalose were likely related to the increase in HSP-27 and the reductions in caspase-3 level. Therefore, trehalose might be a candidate agent to protect against oxidative stress in human neurodegenerative disorders. However, further investigations are warranted to elucidate the underlying mechanisms.

Limitations of the study: In this study, the capase-3 concentration was measured only at mRNA level to investigate the resultant apoptosis. We were not able to measure the caspase-3 activity and other apoptotic markers due to financial constraints.

Recommendations for future research: Future studies are recommended to investigate the effects of trehalose on other critical apoptotic and anti-apoptotic markers to explore the molecular mechanism in support of its neuroprotective capacity.

Ethical Considerations

Compliance with ethical guidelines

The study protocol was reviewed and approved by the Ethics Committee, at Kerman University of Medical Sciences, Kerman (Code: IR.KMU.REC.1399.667).

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Authors' contributions

Experiments and data analysis: Akram Norouzi; Writing, review and editing: Nasrin Ziamajidi; Writing the original drafts: Asieh Sadeghi; Conceptualization and supervision of the study: Mahdieh Nazari-Robati.

Conflict of interest

The authors declare no conflict of interest with any internal or external entities.

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