

Determination of in vitro and in vivo protective effects of Ghrelin against oxidative stress: Experimental Study

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ARTICLE INFO

Article history:

Received on: 08/10/2014

Revised on: 19/10/2014

Accepted on: 12/11/2014

Available online: 29/12/2014

Key words:

Ghrelin, oxidative stress, ROS, HepG2, Rat.

ABSTRACT

Antioxidant properties of ghrelin have been recently reported in animal models and cell culture experiments. This study was set to examine the possible in vitro and in vivo anti-oxidative effects of ghrelin in rat and HepG2 cell culture. In this study, thirty six male Wistar Rats were randomly allocated into six experimental groups of six; Intraperitoneally, group 1 (Control) received 1 ml PBS, group 2 received 0.1 mM/kg tert-Butylhydroperoxide (t-BOOH), groups 3 and 4 received 0.1 mM/kg t-BOOH and then received 10 and 50 µg/kg ghrelin, respectively. Groups 5 and 6 received 10 and 50 µg/kg ghrelin, respectively. Rats were anesthetized 24 h after last injection and blood samples were taken by cardiac puncture. Carbonylated proteins, nitric oxide (NO) and total antioxidant capacity (TAC) levels were measured in sera. HepG2 cells were plateaued at a density of 1.5×10^5 per well in eight plates. After treatment with ghrelin (0, 10, 25 and 50 nM) for 30 min, cells were treated with T-BOOH (100 or 200 µM) for 24 h to analyze cell proliferation by MTT assay at 570 nm. Evidence of oxidative stress including increased carbonylated proteins and NO levels and decreased TAC level were observed after t-BOOH injection. In rats with oxidative stress, subsequent treatment with ghrelin decreased NO and carbonylated proteins level and increased TAC level. The cell viability was decreased after t-BOOH treatment in dose dependent manner; in contrast, ghrelin in all used concentrations caused an elevation in cell viability after 24 h incubation time. These data taken together indicate that ghrelin reduces oxidative stress, but its exact mechanism is yet to be investigated.

INTRODUCTION

In recent years, our understanding of the oxidative stress role in disease etiology has been increased substantially. Oxidative stress is characterized by a disturbance in the balance between reactive oxygen species (ROS) formation, ROS removal and repair of damaged complex molecules. Under certain conditions, production of ROS is enhanced and/or the level or activity of antioxidants is reduced. Oxidative stress is associated

with many human diseases including diabetes mellitus, cardiovascular and neurodegenerative diseases, cancers, liver disease, aging and inflammation (Cederbaum, 2001). ROS are toxic to cells because they can react with most cellular macromolecules causing DNA damage, lipid peroxidation, protein denaturation and enzyme inactivation (McCord, 2004).

Since ROS production is a naturally occurring process, a variety of endogenous enzymatic and nonenzymatic antioxidants have evolved to protect cells against ROS. Antioxidants can be also exogenous compounds acquired from the diet. Antioxidants minimize oxidative damage to biological systems either by preventing formation of ROS, or by quenching ROS before they can react with cellular macromolecules (Alfadda and Sallam, 2012).

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For many years, interest has been focused on strategies that enhance removal of ROS, using antioxidants and drugs that enhance endogenous antioxidants (Day, 2004, McCord, 2004).

Although many pharmacological agents have been effective in the laboratory, several large trials have shown that in some cases they have actually worsened the outcome rather than reducing the destructive events. Recent studies have demonstrated that ghrelin levels are related in disease states associated with oxidative stress (Tesauro *et al.*, 2005). Ghrelin, identified as the main endogenous ligand for growth hormone secretagogue receptors (GHS- R), is a novel 28-amino-acid acylated peptide. It is principally released from gastric cells in the oxyntic mucosa of the stomach. In order to exert its functions, circulating ghrelin needs to activate a 7-transmembrane G protein-coupled receptor (GPCR) called GHS-R. Ghrelin and/or its receptors has been detected in a large number of tissues and cell types including hypothalamus, pancreas, small intestine, kidney, lung, pituitary, brain, placenta, testis and ovary suggesting an endocrine as well as extra-endocrine action of ghrelin (Tesauro *et al.*, 2005, Date *et al.*, 2000). Apart from its effect on energy balance (Horvath *et al.*, 2001), ghrelin has numerous biological actions, such as regulation of cardiovascular actions (Shimizu *et al.*, 2003), modulation of cell proliferation and survival (Baldanzi *et al.*, 2002), inhibition of inflammation and regulation of the immune functions (Şehirli *et al.*, 2008). It has also been reported that ghrelin may be an antioxidant agent. This primary suggestion that ghrelin could inhibit oxidative stress came from the observation that it can inhibit the expression of redox-sensitive genes in endothelial cells, particularly in relation to decreased redox regulated NF-κB activation (Li *et al.*, 2004). It has been proven that ghrelin increases the activity of antioxidant enzymes and decreases the concentration of malondialdehyde (MDA), an end product of lipid peroxidation, in some investigations (Obay *et al.*, 2008, Kawczynska-Drozdz *et al.*, 2006). The similar finding demonstrated that ghrelin decreases formation of ROS and also inhibits vascular superoxide production and oxidative stress in hypertensive rats (Kawczynska-Drozdz *et al.*, 2006). Additional studies have echoed the proposal that ghrelin attenuates the oxidative stress responses (Dong and Kaunitz, 2006).

The protective effect of ghrelin seems to be dependent on different mechanisms. It was suggested that ghrelin leads to release of endogenous nitric oxide (NO). NO is important as a cell signaling molecule, especially involved in vasoconstriction (Guzik *et al.*, 2003). Ghrelin causes vasorelaxation in rats and it improves endothelial function by increasing endothelial NO bioavailability, also NO is a biologically important free radical that regulates a variety of processes in the gastrointestinal tract (Shimizu *et al.*, 2003). As with other free radicals, uncontrolled production of NO can lead to oxidative damage (Ghosh *et al.*, 2004). The mechanisms of these effects are, however, still unknown. In various diseases the endothelium can become dysfunctional and can promote thrombosis and inflammation (Guzik *et al.*, 2003). A major mechanism responsible for such endothelial dysfunction is

excessive production of ROS. ROS can alter production of NO. Therefore, loss of NO bioavailability is a key feature of endothelial dysfunction. Multiple pathways can reduce NO bioavailability by altering its synthesis or biodegradation. Excessive production of ROS seems to be a major mechanism of reduced vascular NO levels (Schächinger *et al.*, 2000).

As mentioned previously ROS can react with most cellular macromolecules like proteins (McCord, 2004). The oxidative modification of proteins by ROS and other reactive compounds is associated with a number of disease and pathophysiological processes (Cederbaum, 2001). What relationships might be among high level of protein carbonyl groups, oxidative stress, and diseases remain uncertain. Under physiological conditions, almost all oxidative modifications of proteins are resulting in an increase of carbonylated proteins. The carbonyl content of proteins is therefore an index of the amount of oxidative protein damage attributable to either direct attack of free radicals or the modification of proteins by oxidation products of carbohydrates or polyunsaturated fatty acids. We have studied the level of protein carbonyl groups as biomarker in the current survey. It has some advantages over other oxidation products because of the relative early formation and the relative stability of carbonylated proteins (Cao and Cutler, 1995, Levine *et al.*, 1994).

Taken together several studies have doubt that ghrelin is associated with oxidative stress (Li *et al.*, 2004), although in the light of the literature knowledge one could hypothesize that ghrelin might prevent or mitigate the effects of oxidative stress. With regard to previous studies, and in order to clarify the possible effect of ghrelin on tert-Butylhydroperoxide (t-BOOH)-induced oxidative stress in rat, the NO and carbonylated proteins content as well as total antioxidant capacity (TAC) as a useful indicator in obtaining a global picture of relative antioxidant activities were measured in the sera of rats. We also conducted in vitro study to elucidate the possible mechanisms, if any, involved in ghrelin's protective effect, by incubating isolated human HepG2 cells with ghrelin to test for its effect on cell viability.

METHODS

In vivo study

Animals and treatment

The project was approved by the committee for ethics in animal experiments of the Mashhad University of Medical Sciences. Male Wistar rats weighting 200-220 g were obtained from the Animal House at Mashhad University of medical sciences, Mashhad, Iran. Rats were maintained on standard rat chow and tap water ad libitum and in an air-conditioned room with a 12 h day/light cycle. All experiments were carried out according to Mashhad University of Medical Sciences ethical committee guidelines and the guidelines of the European Community Union Council for experimental animal care. Every effort was made to minimize animal suffering and the number of animals used.

In this study, 36 male rats, 7-8 weeks of age were divided into six experimental groups (six rats per group) as follows:

- Group 1, received ml PBS (Control),
- Group 2, received 0.1 mM/kg t-BOOH,
- Group 3, received 0.1 mM/kg t-BOOH and 2 h later, injected by 10 µg/kg ghrelin,
- Group 4, received 0.1 mM/kg t-BOOH and 2 h later, injected by 50 µg/kg ghrelin,
- Group 5, received 10 µg/kg ghrelin, and
- Group 6, received 50 µg/kg ghrelin.

Ghrelin and t-BOOH were purchased from innovagen (Sweden), dissolved separately in sterile PBS and injected intraperitoneally (i.p.). All the injections were carried out between 8 to 12 AM.

At the end of the experiment (i.e. 24 h after the last injection), rats were anesthetized by i.p. injection of urethane at a dose of 125 mg/100 g bodyweight. Then 8-10 ml blood sample was taken by cardiac puncture. After centrifugation at 3000 g for 10 min at 4 °C, the sera were separated. Sera were stored at -20°C for determination of NO, TAC and carbonylated proteins.

Measurement of NO level in serum

The amount of NO was determined by measuring the concentration of nitrite, a metabolite of NO, using a modified Griess reaction method (Green *et al.*, 1982). Briefly the prepared sera mixed with an equal volume of modified Griess reagent for the colorimetric assay.

After 10 min incubation at room temperature, the concentration of the resultant chromophore was measured spectrophotometrically at 550 nm after enzymatic conversion of the nitrate to nitrite by nitrate reductase. The nitrite concentration in the samples was calculated from nitrite standard curves made from sodium nitrite using the same sera.

Measurement of TAC in serum

The TAC of serum was determined by measuring its ability to reduce Fe³⁺ to Fe²⁺ using the FRAP test as described previously (Benzie and Strain, 1999). Briefly, the working Ferric Reducing Ability of Plasma (FRAP) reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4, 6-tripyridyl-s-triazine (TPTZ) solution (10 mM TPTZ in 40 mM HCl) and 20 mM FeCl₃.6H₂O in a 10:1:1 ratio just before use and heated to 37°C. H₂O-diluted sample was then added to 300 µl freshly prepared reagent warmed at 37 °C. The absorption of the blue color complex was recorded at 593 nm by Tecan Sunrise Microplate Reader (Tecan, Austria).

Measurement of carbonylated proteins in serum

As slightly modified version of the method, originally developed by Levine *et al.* (Levine *et al.*, 1994), was used in this study for the specific detection of carbonylated proteins (oxidized proteins). Briefly, firstly the protein carbonyls are derivatized by

2,4-DNPH to yield the corresponding 2,4-dinitrophenyl hydrazones.

In a second step, the dinitrophenyl (DNP) groups associated with proteins, are detected immunochemically using a commercial anti-DNP antiserum (Invitrogen) and conventional peroxidase staining system.

In vitro study

Cell culture and ghrelin treatment

Human hepatoma HepG2 cells were purchased from Institute Pasteur (Tehran, Iran) and cultured in Dulbeccos modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin under an atmosphere of 95% air and 5% CO₂ at 37°C. Prior to any treatment, cells were starved in serum-free DMEM for 24 h. For all experiments, the cells were grown to approximately 60-80% confluence.

HepG2 cells were plated at a density of 1.5 x 10⁵ per well in eight plates as; plate 1 treated with 100 µM t-BOOH (control-1), plate 2 treated with 10 nM grehlin and 100 µM T-BOOH, plate 3 treated with 25 nM grehlin and 100 µM T-BOOH, plate 4 treated with 50 nM grehlin and 100 µM T-BOOH, plate 5 treated with 200 µM T-BOOH (control-2), plate 6 treated with 10 nM grehlin and 200 µM T-BOOH, plate 7 treated with 25 nM grehlin and 200 µM T-BOOH and plate 8 treated with 50 nM grehlin and 200 µM T-BOOH.

After treatment with or without ghrelin for 30 min, cells were treated with T-BOOH for 24 h to analyze cell viability. Incubation media were not changed during this time. After incubation period, media were removed, centrifuged and frozen until laboratory measurements. Cells were then trypsinized and used for viability assay.

Cell viability assay

After all treatments, Cell viability was assessed by MTT as described previously (Baldanzi *et al.*, 2002). Cells were seeded on 96-well plates at a density of 1.5 x 10⁵ cells per well. For detection, cells were incubated with 1 mg/mL MTT for approximately 1 h.

The medium was removed and the formazan product was solubilized with 150 µL dimethylsulfoxide. Viability was assessed by spectrophotometry at 570 nm absorbance using a 96-well plate reader.

Statistical analysis

Statistical analysis was performed using the SPSS for Windows statistical package, version 16 .0. All values are expressed as the mean±SD. Statistical significance of differences was determined using analysis of variance (one-way ANOVA). Further statistical analysis for post-hoc comparisons was carried out using the Tukey-Kramer multiple comparison tests. A level of p<0.05 was considered statistically significant.

RESULTS

In vivo studies

A single i.p. injection of t-BOOH at a dose of 0.1 mM/kg in group 2 induced oxidative stress approximately 2 hours after injection. Evidence of oxidative stress, which included increased carbonylated proteins and NO levels and decreased TAC level were observed in this group (Figures 1–3).

Determination of carbonylated proteins in serum

Carbonylated proteins levels, the indicator of the amount of oxidative protein damage, were increased in the serum of T-BOOH -induced oxidative stress rats (8.8 ± 0.99 nM) when compared with the controls (5 ± 1.22 nM). Ten and fifty $\mu\text{g}/\text{kg}$ doses of ghrelin injection after 2 h prevented this elevation. A single injection of ghrelin at doses of 10 and 50 $\mu\text{g}/\text{kg}$ reduced carbonylated proteins level (groups 5 and 6, respectively) when compared to groups 1 and 2. The differences between groups 2 and 6 was significant ($p=0.019$) (Figure 1).

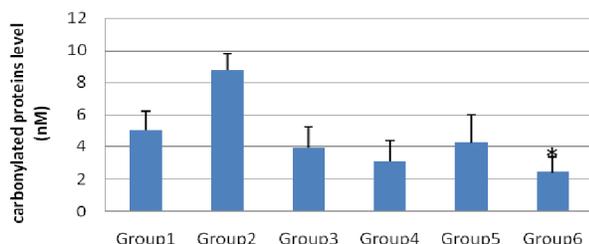


Fig. 1: Effect of different concentrations of gherin on carbonylated proteins level in serum of rats (Mean \pm SD). **Group 1** (Control): 1 ml PBS, **Group 2:** 0.1 mM/kg tert-Butylhydroperoxide (t-BOOH), **Group 3:** 0.1 mM/kg t-BOOH + 10 $\mu\text{g}/\text{kg}$ ghrelin, **Group 4:** 0.1 mM/kg t-BOOH + 50 $\mu\text{g}/\text{kg}$ ghrelin, **Group 5:** 10 $\mu\text{g}/\text{kg}$ ghrelin and **Group 6:** 50 $\mu\text{g}/\text{kg}$ ghrelin. * significant differences with group 2.

Determination of NO level in serum

T-BOOH injection increased NO level in group 2 (28.1 ± 4.81 μM) as compared to the control group (23.2 ± 2.91 μM). Treatment with ghrelin in groups 3 and 4 after 2 h abrogated the elevation induced by t-BOOH as compared to the group 2. Besides, single ghrelin treatment in groups 5 and 6 increased NO content (30.1 ± 5.04 and 28.7 ± 2.32 μM , respectively) in comparison to the control level (23.2 ± 2.91 μM). The difference between groups 1 and 5 was significant ($p=0.044$) (Figure 2).

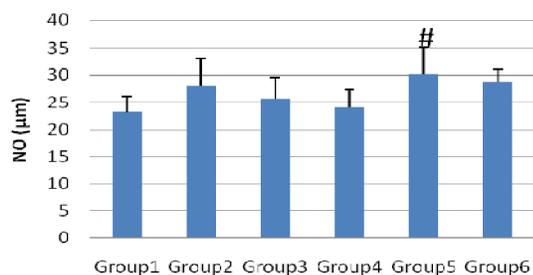


Fig. 2: Effect of different concentrations of gherin on NO level in serum of rats (Mean \pm SD). **Group 1** (Control): 1 ml PBS, **Group 2:** 0.1 mM/kg tert-Butylhydroperoxide (t-BOOH), **Group 3:** 0.1 mM/kg t-BOOH + 10 $\mu\text{g}/\text{kg}$ ghrelin, **Group 4:** 0.1 mM/kg t-BOOH + 50 $\mu\text{g}/\text{kg}$ ghrelin, **Group 5:** 10 $\mu\text{g}/\text{kg}$ ghrelin and **Group 6:** 50 $\mu\text{g}/\text{kg}$ ghrelin. # significant difference with group 1.

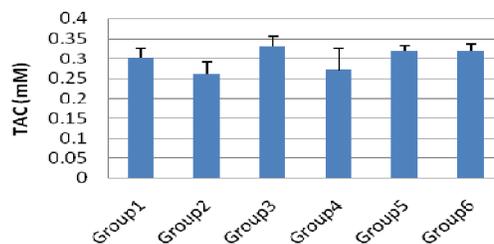


Fig. 3: Effect of different concentrations of gherin on TAC level in serum of rats (Mean \pm SD). **Group 1** (Control): 1 ml PBS, **Group 2:** 0.1 mM/kg tert-Butylhydroperoxide (t-BOOH), **Group 3:** 0.1 mM/kg t-BOOH + 10 $\mu\text{g}/\text{kg}$ ghrelin, **Group 4:** 0.1 mM/kg t-BOOH + 50 $\mu\text{g}/\text{kg}$ ghrelin, **Group 5:** 10 $\mu\text{g}/\text{kg}$ ghrelin and **Group 6:** 50 $\mu\text{g}/\text{kg}$ ghrelin.

Determination of TAC level in serum

TAC was 0.26 ± 0.031 mM in the group 2 which received t-BOOH when compared with the control group (0.3 ± 0.026 mM). Ghrelin treatment produced an elevation in TAC content up to 0.33 ± 0.025 mM and 0.27 ± 0.057 mM in groups 3 and 4, respectively. The similar elevation was observed in groups 5 and 6 (0.32 ± 0.013 mM and 0.32 ± 0.017 , respectively) when compared to others. There were not significant differences among groups (Figure 3).

In vitro studies

Ghrelin promotes HepG2 cells viability

As shown in Figure 4, cell viability was decreased after t-BOOH treatment in a dose dependent manner; the single dose of 200 μM (group 5) was more effective than 100 μM (group 1). Ghrelin in all used concentrations (10, 25 and 50 nM) caused an elevation in cell viability after 24 h incubation time and promoted cell growth as compared with groups 1 and 5, most effectively by 50 nM, 10 nM being the lowest active concentration.

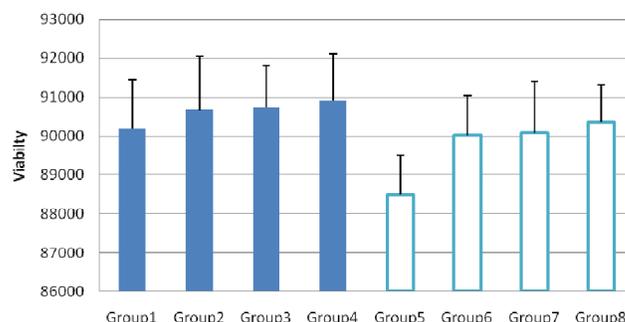


Fig. 4: Effects of gherlin on viability of HepG2 cells. **Group 1:** 100 μM t-BOOH (control-1), **Group 2:** 10 nM ghrelin and 100 μM T-BOOH, **Group 3:** 25 nM ghrelin and 100 μM T-BOOH, **Group 4:** 50 nM ghrelin and 100 μM T-BOOH, **Group 5:** 200 μM T-BOOH (control-2), **Group 6:** 10 nM ghrelin and 200 μM T-BOOH, **Group 7:** 25 nM ghrelin and 200 μM T-BOOH and **Group 8:** 50 nM ghrelin and 200 μM T-BOOH.

DISCUSSION

In the present study, t-BOOH was used for inducing oxidative stress. T-BOOH, a membrane-permeate oxidant that has been extensively used as a model of oxidative stress in different systems (Garcia-Cohen *et al.*, 2000). Our data showed that oxidative stress can be induced in 2 hour by a single i.p. injection of t-BOOH at a dose of 0.1 mM/kg. Evidence of oxidative stress,

which included increased carbonylated proteins and NO as well as decreased TAC level were observed after t-BOOH injection. ROS production induced upon t-BOOH treatment is the major contributors to cell damage and involved in a variety of biological phenomena.

The most prominent characteristic of ROS is their high reactivity with other molecules (Alfadda and Sallam, 2012). In normal conditions, cells are protected from the ROS by antioxidant defense, involving oxygen scavenger enzymes as well as vitamins E and C (Zadák *et al.*, 2009).

The current study demonstrates that administration of ghrelin produced a protective effect against oxidative stress in the rat. Our data showed that the protective effect of ghrelin is mediated by its antioxidant promoting activity *in vivo* as determined by an elevation in the level of TAC. The mechanism by which ghrelin inhibited ROS and oxidative stress is probably through blocking of some of the enzymes needed for their production and also its ability to inhibit the expression of redox sensitive pro-inflammatory genes (Li *et al.*, 2004). Nevertheless influence of ghrelin on antioxidant level is still unknown. Previous investigators have shown that ghrelin treatment significantly increased antioxidant activities and reduced formation of ROS and MDA levels in rats (İşeri *et al.*, 2005). Although there is some study in contrast with our findings, illustrated incubating ghrelin in the xanthine-xanthine oxidase reaction did not have any inhibitory effect on ROS formation (Li *et al.*, 2004). This suggests that *in vivo* effects of ghrelin may be exerted through other mechanisms rather than the xanthine-xanthine oxidase.

In the present study, administration of ghrelin decreased the serum level of NO in rats treated with t-BOOH. The mechanisms involved in the proinflammatory actions of NO are unclear but may involve the function of NO as a free radical and/or conversion of NO to more reactive nitrogen species that can induce cell damage (Miller and Sandoval, 1999). We found that single injection of ghrelin appears to mediate the up-regulation of NO in rats. Previous studies showed that ghrelin increases NO production in a number of ways. Xu *et al.*, showed that ghrelin activated eNOS in cultured endothelial cells and intact vessels (Xu *et al.*, 2008).

Therefore, ghrelin may act directly within the vascular wall, leading to the improvement of NO bioavailability in animal models and in humans. The potential mechanisms of this improvement remain unknown, though it can be mediated through S-nitrosylation of cellular proteins which can be regarded as a protective mechanism against the irreversible oxidation of these macromolecules (Xu *et al.*, 2008, Miller and Sandoval, 1999).

Protein carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation, and accumulation of protein carbonyls has been observed in several human diseases (Cao and Cutler, 1995, Levine *et al.*, 1994). The usage of protein Carbonyl (CO) groups as a marker of oxidative stress may have some advantages including early formation and longer stability in the blood of patients compared with other parameters of oxidative stress. The

relationship among protein oxidation, protein dysfunction, and diseases remains largely unclear; however, it is known that oxidative modification of enzymes and structural proteins may play a significant role in the etiology of diseases. Oxidative damage often leads to loss in specific protein function and structure (Pantke *et al.*, 1999).

Our findings illustrated that CO groups' content increased in the serum of T-BOOH -induced oxidative stress rats. CO groups are produced on protein side chains when they are oxidized (Levine *et al.*, 1994). We determined the reducing effects of ghrelin on CO groups' content in current survey and the mechanism of this antioxidant effect is still not clear. Further experimental studies and appropriate clinical studies are needed to elucidate the exact mechanism.

The *in vitro* study provided evidences that ghrelin inhibited cell death in a dose-dependent manner, suggesting the antioxidant activity of ghrelin as a potential mechanism for its protective effect. The current *in vitro* study supported our *in vivo* data that ghrelin, via improving antioxidant capacity, promotes cell viability.

Similar data was presented by other authors. Kim *et al.* showed that ghrelin at different concentrations increased adipocyte cell number in 3T3-L1 culture cells (Kim *et al.*, 2004). Another study demonstrated that ghrelin promotes β -cell survival and partially inhibited apoptosis induced by lipotoxicity in MIN6 cells (Wang *et al.*, 2010). Further studies should be carried out to investigate the effect of ghrelin administration on oxidative stress status in cell culture.

Possibly, prolonged treatment by ghrelin or higher doses is needed to induce greater activity of antioxidant. In the light of the literature knowledge and our data, one could hypothesize that the protective effect of ghrelin on t-BOOH -induced oxidative stress status depends on its antioxidant properties. Further researches are necessary to confirm this hypothesis. These data taken together indicate that ghrelin reduces oxidative stress, but the mechanism of ghrelin's antioxidant effect is still not clear.

CONCLUSION

Current findings may help to explain the beneficial effects of ghrelin administration in various pathophysiological conditions associated with oxidative stress and encourage the use of ghrelin as an approach in the prevention and/or treatment of such condition. In rats with oxidative stress, treatment with ghrelin decreased NO and CO level, increased TAC level and also promoted cell growth in HepG2 cell culture. In conclusion, the present study provides the evidence and potential mechanism of protective effect ghrelin against oxidative stress.

ACKNOWLEDGMENTS

This study was financially supported by Research Vice-Chancellery of Mashhad University of Medical Sciences, Iran. This paper is a part of Mahdi Nazoury's MSc thesis (research project No. 900702).

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How to cite this article:

Mahdi Nazoury, Seyed-Mostafa Hosseini-Zijoud, Mohammad Soukhtanloo, Baratali Mashkani, Seyed Isaac Hashemy. Determination of in vitro and in vivo protective effects of Ghrelin against oxidative stress: Experimental Study. *J App Pharm Sci*, 2014; 4 (12): 008-013.