International Journal of Pharmacy Teaching & Practices 2013, Vol.4, Issue 1, 451-457.

Antioxidant enzyme activities assay and thiobarbituric acid reactive substances concentration following administration of ghrelin in the rat kidney

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Research Article

Please cite this paper as: Shima Neamati [*1], Masoud Alirezaei [2], Arash Kheradmand [3], Marzieh Rashidipour [4], Mohammad Reza Saki [5]. Antioxidant enzyme activities assay and thiobarbituric acid reactive substances concentration following administration of ghrelin in the rat kidney. IJPTP, 2013,4(1), 451-457.

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Abstract

Background: The aim of the present study was to examine the possible antioxidant properties of ghrelin by evaluation of enzymatic (glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase, (CAT)) and non-enzymatic (GSH-content) antioxidant defense system, as well as determination of lipid peroxidation marker (thiobarbituric acid reactive substances, TBARS) in the rat kidney.

Method: Twenty-eight adult Wistar rats (male and female equally) were subdivided into control and treatment groups. Treatment groups received 3 nmol of ghrelin as subcutaneous injection for 10 consecutive days while, physiological saline injected to the control groups. The rats were killed one the day after the last treatment and their kidneys were taken for biochemical analysis.

Results: Catalase activity was significantly higher in the ghrelin-treated rats when compared to the controls for both genuses (P <0.05). In contrast, lipid peroxidation value, (TBARS), reduced significantly on both genuses in the ghrelin-exposed animals (P <0.05). Furthermore, the mean activity of SOD and GSH-content were significantly higher in the female ghrelin-treated rats compared to the controls but, these differences were not statistically significant for male ghrelin-treated rats.

Conclusion: Our findings suggest that ghrelin possesses possibly beneficial antioxidant effects in the kidney of rats, via enhancing activities of CAT, and SOD subsequently, increasing GSH-content and GPx activity, respectively. These effects were noticeable in the females than to the males possibly, due to the higher level of oestrogen.

Keywords: *Rat kidney, Antioxidant enzymes, Ghrelin, TBARS, GPx.*

Introduction

Ghrelin has been identified as an endogenous ligand for growth hormone secretagogue receptor (GHSR) that regulates growth hormone secretion, regulates food intake, increases appetite and contributes to insulin release and energy homeostasis [1, 3, 10, 11]. It has been detected in a large number of tissues and cell types, including hypothalamus, small intestine, pancreas, placenta, pituitary, brain, lung, and kidney [12-14]. There have also been reports on the renal protective effects of growth hormone (GH) and insulin growth factor-1 (IGF-1) against various types of renal damage [15]. As for the renal protective mechanism of ghrelin, induction of nitric oxide (NO) and cGMP in the kidney by GH and IGF-1 were suggested to improve renal circulation [12, 16, 17]. Otherwise, in recent years more evidence support the hypothesis that ghrelin may be an antioxidant and anti-inflammatory agent. For instance, very recently we proved that ghrelin prevents lipid peroxidation and reduction of antioxidant enzymes activities (Glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT)) and glutathione content (GSH-content) in the rat testes and ovaries [3, 11, 18]. Also, there are other reports about protective effects of ghrelin against pentylenetetrazole-induced oxidative stress in the erythrocytes, liver and brain [1], and burn-induced multiple organ injury in rats [19].

The excessive production of reactive oxygen species (ROS) in the human body is involved in the pathogenesis of various diseases including atherosclerosis, diabetes mellitus, stork, inflammatory diseases and end-stage renal disease (ESRD) [1, 2]. ROS, such as superoxide anion, hydrogen peroxide, and hydroxyl radical, are generated as byproducts of oxidative metabolism in mitochondria, can interact with biomolecules such as DNA, RNA, protein, and lipids subsequently, damaging various cellular components [3-6]. Oxidative stress can also influence insulin sensitivity of peripheral tissues [2]. In this regard, ESRD is

associated with a state of insulin resistance [7]. Interestingly, inflammation has been demonstrated to derange mitochondrial function thereby favoring leakage of ROS [2, 8]. It seems that, inflammation either directly, that is, activating the proteolytic systems, or indirectly, that is, favoring the development of oxidative stress and insulin resistance, is the main mediator of wasting in diseases with chronic renal failure such as ESRD, also considering its role in anorexia [9].

In the light of these reports one could hypothesize that ghrelin might increase antioxidant status of the kidney. Thus, we undertook this study to examine whether exogenous ghrelin has antioxidant effects on the rat kidney. In order to clarify the possible antioxidant effects of ghrelin, the activities of main antioxidant enzymes (GPx, SOD, CAT), and GSH-content and lipid peroxidation level were measured in the kidney of rats.

Material and Method

Drugs and chemicals

Rat lyophilised acylated ghrelin (n-octanoylated research grade) was purchased from Tocris Cookson Ltd. (Bristol, UK). Ghrelin was dissolved in sterile physiologic saline solution before injection. The kits used in the measurement of antioxidant enzymes activities were provided from Randox Laboratories Ltd. (Antrim, UK). Other chemicals were purchased from Sigma-Aldrich Company (St. Louis, MO.USA) unless otherwise indicated.

Animals

Thirty-eight adult Wistar rats (males=14 and females=14, weighing 220–250 g) obtained from Animal House Center, Shiraz University of Medical Sciences, Shiraz, Iran and were housed in temperature-controlled conditions under a 12:12-h light/dark photocycle with food and tape water supplied *ad libitum.* All rats were treated humanely and in compliance with the recommendations of Animal Care Committee for the Lorestan University of Medical Sciences (Khorram Abad, Iran). All of experimental procedures were carried out between 10.00 -12.00 am.

Experimental design

The rats were divided into four equal groups, as two controls (male or female, each group containing 7 rats) and treatment groups. The rats treated daily for 10 consecutive days as following order: the control groups received physiological saline (100 µl) in order subcutaneous (S.C.) injection once a day and the treatment groups were injected ghrelin (3 nmol/100 µl saline, S.C.). The dose used in our study, is similar to the physiological concentration of circulating ghrelin in the fasting state of rats [13, 20]. The animals were injected under conscious conditions after careful handling to avoid any stressful influence. One the day after the last injection, the rats were killed upon diethyl ether anesthesia (Dagenham, UK) by decapitation. Immediately after rat killing, right kidneys were removed and carefully cleaned of fat and adhering and stored at −70 °C for biochemical analysis.

Analysis and measurements

Tissue preparation for protein measurement and enzyme assay

Rat kidneys were rapidly thawed and manually homogenized in cold phosphate buffer (0.1 mmol/lit, pH 7.4, containing 5mmol/lit EDTA) and debris removed by centrifugation at 2000 g for 10 min (Centrifuge 5415 R; Rotofix 32A, Germany). Supernatants were recovered and used for protein measurement, lipid peroxidation value, antioxidant enzyme activities, and GSH-content. Protein content of kidneys supernatants was determined using a colorimetric method of Lowry with bovine serum albumin as a standard [21].

Measurement of lipid peroxidation

The concentration of lipid peroxidation was indicated by the content of thiobarbituric acid reactive substances (TBARS) in the kidney. Tissue TBARS determined by following the production of thiobarbituric acid reactive substances as described previously [22]. In short, 40 µl of supernatant was added to 40 µl of 0.9% NaCl and 40 µl of deionized H_2O, resulting in a total reaction volume of 120 µl. The reaction was incubated at 37º C for 20 min and stopped by the addition of 600 µl of cold 0.8 mol/l hydrochloride acid, containing 12.5% trichloroacetic acid. Following the addition of 780 µl of 1% TBA, the reaction was boiled for 20 min and then cooled at 4º C for 1 h. In order to measure the amount of TBARS produced by the homogenate, the cooled reaction was spun at 1500 *g* in a microcentrifuge for 20 min and the absorbance of the supernatant was spectrophotometrically read at 532 nm, using an extinction coefficient of 1.56×10^5 /M.Cm. The blanks for all of the TBARS assays contained an additional 40 µl of 0.9% NaCl instead of homogenate as just described. TBARS results were expressed as nmol per milligram of tissue protein (nmol/mg protein).

Measurement of CAT activity

Tissue catalase activity was assayed using the method described by Claiborne [23]. The reaction mixture (1 ml) consisted of 50 mmol/lit potassium phosphate (pH 7.0), 19 mmol/lit H_2O_2, and a 25 µl sample. The reaction was initiated by the addition of H_2O_2 and absorbance changes were measured at 240 nm (25 °C) for 30 s. The molar extinction coefficient for H_2O_2 is 43.6/M.Cm. The CAT activity was expressed as the unit that is defined as µmol of H_2O_2 consumed per min per milligram of tissue protein (unit/mg protein).

Measurement of SOD activity

The activity of superoxide dismutase (SOD) was evaluated with Randox SOD detection kit according to the manufacturer's instructions. The role of SOD

is to accelerate the dismutation of the toxic superoxide (O_2^-) produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The SOD activity is then measured by degree of inhibition of this reaction. One unit of SOD is that which causes 50% inhibition of the rate of reduction of INT under the conditions of the assay. SOD levels were recorded at 505 nm and through a standard curve and expressed as unit per milligram of tissue protein (unit/mg protein).

Measurement of GPx activity

The activity of glutathione peroxidase (GPx) was evaluated with Randox GPx detection kit according to the manufacturer's instructions. GPx catalyse the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidised glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance was measured spectrophotometrically (S2000 UV model; WPA, Cambridge, UK) against blank at 340 nm. One unit (U) of GPx was defined as l µmol of oxidized NADPH per min per milligram of tissue protein. The GPx activity was expressed as unit per milligram of tissue protein (unit/mg protein).

Total glutathione (GSH content)

Total glutathione was estimated by the model as described previously [24]. In brief, 5% tissue supernatants prepared in 20 mmol/lit EDTA, pH 4.7, and 100 µl of the supernatant or pure GSH was added to 0.2 mol/lit Tris–EDTA (1.0 ml, pH 8.2) buffer (Fluka, Switzerland) and 20 mmol/lit EDTA, pH 4.7 (0.9 ml) followed by 20 µl of Ellman's reagent (10 mmol/lit DTNB in methanol). After 30 min of incubation at room temperature, absorbance was read at 412 nm. The blank was prepared with the same method however, instead of 100 µl of the tissue supernatant, 100 µl of distilled water was applied. Both the blank and sample reaction mixtures were read against water at 412 nm. GSH concentration was calculated on the basis of a millimolar extinction coefficient of 13.6 and a molecular weight of 307 g.

Statistical analysis

All results are presented as mean± (S.E.M.). The statistical differences were assed between the control and ghrelin-treated rats in each sense (males or females) by an Independent-sample t-test (Graphpad PRISM version 5; Graphpad Software Inc., San Diego, CA, USA). Previously, all variables were tested for normal and homogeneous variances by *leven´s statistic* test. *P* value of less than 0.05 was considered to be statistically significant.

Results and Discussion

The mean± S.E.M. values of TBARS concentration, CAT and SOD activities, GSH-content as well as GPx activity in the kidney of rats are shown in figures 1-5, respectively. TBARS concentrations (as lipid peroxidation marker), were significantly lower in the ghrelin-treated rats compared to the control rats (*P* <0.05). In contrast, administration of ghrelin to rats for 10 consecutive days significantly increased the activity of CAT on both experimental genuses in the ghrelin-treated rats compared to the control groups (*P* <0.05). Although, ghrelin could enhance significantly the mean activity of SOD and GSH-content in the female ghrelin-treated rats, but the increased activity of SOD and GSH-content were not statistically significant in the male ghrelin-exposed rats. However, in GPx activity for both experimental genuses in the ghrelin-treated animals similar increasing were observed but, these enhancements were not statistically significant (*P* <0.05).

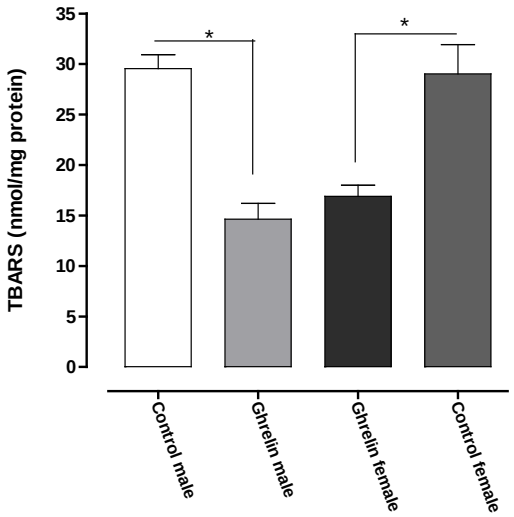

Fig 1. Effect of ghrelin on thiobarbituric acid reactive substances concentration (TBARS nmol/mg protein of kidney) in the control and ghrelin-treated rats. Star indicates statistical difference between groups (*P* <0.05).

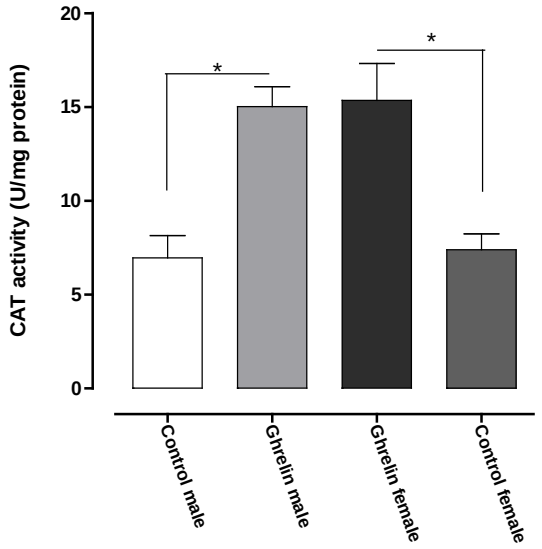

Fig 2. Effect of ghrelin on catalase activity (CAT unit/mg protein of kidney) in the control and ghrelin-treated rats. Star indicates statistical difference between groups ($P < 0.05$).

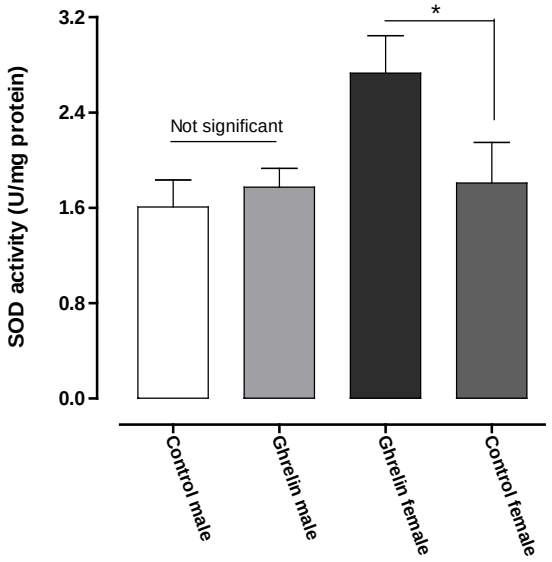

Fig 3. Effect of ghrelin on superoxide dismutase activity (SOD unit/mg protein of kidney) in the control and ghrelin-treated rats. Star indicates statistical difference between females groups ($P < 0.05$) while, there is not statistical difference between males groups ($P > 0.05$).

Many patients with end-stage renal disease (ESRD) are malnourished, partly because of loss of appetite [25]. Ghrelin is a potent orexigen that increases food intake, while it leads to a dose-dependent inhibition of cytokine expression, suggesting that ghrelin may couple the metabolic axis to the immune system, which supports the potential use of ghrelin in the management of disease-associated cachexia [26]. Ashby *et al.* (2009) studied the effect of ghrelin administration on 12 malnourished dialysis patients for 1 week. The administration of ghrelin increased its concentration in the blood for 2 hours.

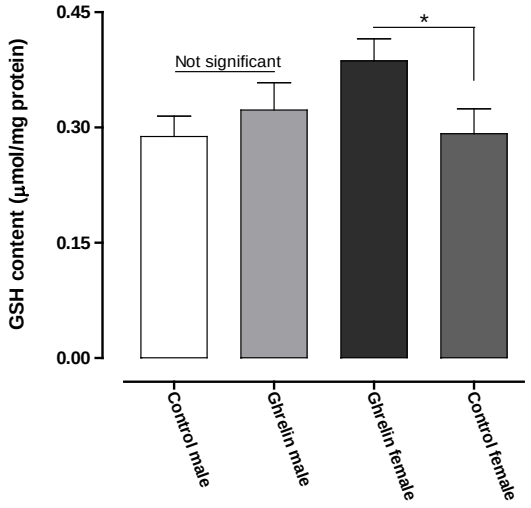

Fig 4. Effect of ghrelin on glutathione content (GSH-content µmol/mg protein of kidney) in the control and ghrelin-treated rats. Star indicates statistical difference between females groups ($P < 0.05$) while, there is not statistical difference between males groups ($P > 0.05$).

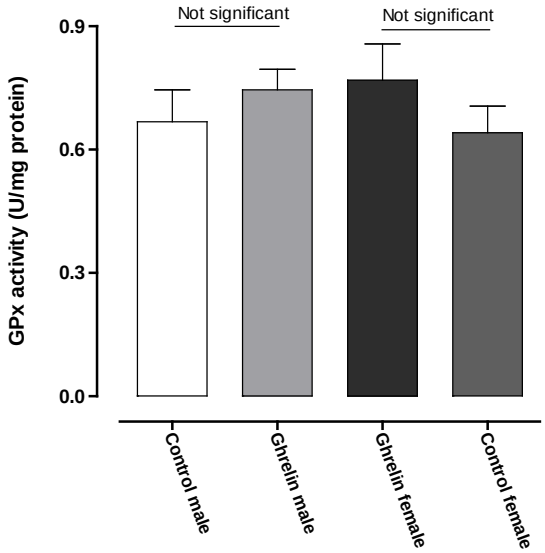

Fig 5. Effect of ghrelin on glutathione peroxidase activity (GPx unit/mg protein of kidney) in the control and ghrelin-treated rats. There is no statistical difference between groups ($P > 0.05$).

Also, it immediately increased appetite and energy intake for the first meal after injection [27]. It is well known that physiologic activity of ghrelin is mediated by an interaction between ghrelin and growth hormone secretagogue receptor (GHSR) [12]. Recently, several groups reported that GHSR existed in the pituitary, myocardium, aorta, and kidney and that various tissues, including the kidney, expressed the ghrelin gene [12, 28]. Otherwise, Mori *et al.* (2000) reported that ghrelin was produced locally in the kidney, suggesting a direct effect of ghrelin on the

kidney [29]. However, in this study we could to show an increasing of antioxidant status in the kidney of rats by treatment with ghrelin. In this regard, lipid peroxidation process (Shown as TBARS concentration) significantly decreased in both genuses of ghrelin-treated rats. In this study, we showed that ghrelin elevates renal antioxidant status and decreased renal lipid peroxidation in the Wistar rats. These beneficial effects of ghrelin were associated with enhancement of CAT and SOD activities subsequently, decreasing lipid peroxidation process. The present study demonstrated for the first time that chronic administration of ghrelin can promote antioxidant enzymes activities and GSH-content particularly in females, suggesting a promising synergist antioxidant effect between ghrelin and oestrogen.

Gambineri *et al.* (2003) evidenced that androgens are independent modulators of ghrelin levels in women, thus confirming an interaction between ghrelin and oestrogen synthesis [30]. For example, it has been indicated that produced ghrelin by the ovarian follicles or physiological doses of ghrelin in the cultured ovarian follicles stimulated estradiol secretion and increased aromatase activity, as a key enzyme in oestrogen biosynthesis [31, 32]. Oestrogen may regulate ghrelin secretion by a positive feedback mechanism. Evidence for this is that the plasma ghrelin concentrations during the follicular phase of the menstrual cycle in women are greater than the men [33]. Thus, it seems, higher oestrogen concentration in the female rats acts as a synergist antioxidant agent with ghrelin. In this sense, it is established that oestrogen is one of the non enzymatic antioxidant in the ovary, previously [3, 34].

Ghrelin was shown to protect the kidneys from ischemia/reperfusion injury [12]. Although, the most rational dosage of ghrelin is still unclear, in this study we injected ghrelin (3 nmol/rat) to examine whether this therapeutic regimen for 10 consecutive days is capable to increase renal antioxidant defense system. This injection schedule was based on the ghrelin concentration in the fasting state of rats [13], and 1.5 fold higher than the dose was used in our recent study for evaluation of antioxidant enzymes in rat ovary [3]. We think that only one injection is not sufficient to protect renal function from oxidative stress and the treatment protocol that was used in our study and others is appropriate to protect against oxidative damage during the reperfusion period in renal failure [12, 35].

The result of oxygen radical formation is damage to an array of biomolecules found in tissues, including nucleic acids, membrane lipids, enzymes, and receptors [26]. Membrane-associated polyunsaturated fatty acids are readily attached by hydroxyl radical in a process that leads to peroxidation of lipids, which can disrupt membrane fluidity and cell compartmentation [26, 36]. It has been clearly indicated that the lipid peroxidation significantly increases by accumulation of H_2O_2 in a concentration-dependent manner [37]. Cells are able to defend themselves from damaging effects of oxygen radicals by way of their own antioxidant mechanisms, including enzymatic and non-enzymatic antioxidant systems [19, 38]. GPx and CAT are two key antioxidant enzymes that can decompose hydrogen peroxide to water [4, 11]. SOD, another antioxidant enzyme in cells rapidly converts superoxide anion (O^{2-}) to less dangerous hydrogen peroxide (H_2O_2) and then GPx and CAT can decompose H_2O_2 to water [3, 18]. Although, H_2O_2 is not a particularly reactive product, it may be reduced to the highly reactive metabolites hydroxyl radicals and/or single oxygen [39]. In addition, reduced glutathione (GSH) is a tripeptide that is present in all mammalian cells and has a major cytoprotective function as a reductant and co-factor for certain antioxidant enzymes such as GPx and this function of GSH is of particular importance in defense against oxidative stress [1, 40]. In this sense, GSH and other antioxidants play a critical role in limiting the propagation of free radical reactions, which would otherwise result in extensive lipid peroxidation [19]. In the present study, ghrelin caused significant increases in the catalase activity for both genuses and SOD activity as well as GSH-content for only female rats, while ghrelin treatment abolished the increase in TBARS concentration probably, in part, by scavenging the very reactive oxygen derived free radicals. On the other hand, renal GSH content was not depleted in the female ghrelin-treated rats, suggesting that ghrelin with concomitant oestrogen possessed antioxidant effects and preserved the cellular antioxidant stores. In accordance with renal GSH result, other antioxidant enzymes were increased in female ghrelin-treated rats except GPx.

Although, the mean activity of GPx in our study was slightly higher in the ghrelin-treated rats than the controls however, these differences were not statistically significant. Possibly, prolonged treatment by ghrelin or higher doses is needed to induce greater activity of GPx in the kidney. On the other hand, as indicated in the figure 5, GPx seems to be less affected by ghrelin and this hormone could not markedly influence renal GPx activity. Possibly, one of the reasons for unaffected GPx activity may be due to depletion of GSH-content, particularly in renal male treated rats. The increase in SOD activity in our investigation for female rats correlates well with the increase of GSH content and the decrease in lipid peroxidation in the renal tissue. SOD shifts highly reactive O^{2-} to H_2O_2 and thus prevents the renal cell membrane damage caused by this highly toxic anion. In this regard, Kawczynska-Drozdz *et al.* (2006) showed that ghrelin is able to directly inhibit vascular superoxide production in spontaneously hypertensive rats and decrease blood pressure [26, 41]. Furthermore, in vitro studies verified that ghrelin increases the mRNA levels of superoxide dismutase in fish leukocytes, implicating an anti-oxidant mechanism [42]. Our findings indicate that ghrelin reduces superoxide radical production and therefore decreases formation of lipid peroxidation products. Similar results were also found in our recent studies, in which ghrelin increased antioxidant enzymes activities and reduced lipid

peroxidation in the rat ovaries and testes following chronic administration of ghrelin [3, 18].

Conclusion

These data and those in the literature suggest that ghrelin with its antioxidant and anticataolic effects may have potential benefits in the management of ESRD and support the use of exogenous ghrelin as a potential therapy for renal oxidative stress-induced diseases including; cachexia and ESRD.

Acknowledgement

This research was financially supported by Islamic Azad University, Brojerd Branch, Young Researchers Club, Lorestan, Iran. We are most grateful to Dr. B. Rasoulian for his kindly cooperation in Razi Herbal Medicines Research Center, Lorestan University of Medical Science, Khorram Abad, Iran.

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AUTHORS' CONTRIBUTIONS

Authors contributed equally to all aspects of the study.

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Not commissioned; externally peer reviewed.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.