Antioxidant properties of ghrelin have been recently reported on various oxidative stresses in limited tissues. This study was set to examine the possible antioxidative effects of ghrelin in rat ovarian tissue. Twenty eight female adult Wistar rats were randomly allocated into control and treatment groups. Treatment group ($n=14$) received $2\text{ nmol}$ of ghrelin as subcutaneous injection for 14 consecutive days or vehicle (physiological saline) to the control rats. The animals from both groups were equally killed on days 9 and 14 after beginning of ghrelin injection ($n=7$ from each group on each day) and their ovaries were taken for later antioxidant enzyme activity assays as well as measurement of glutathione content and thiobarbituric acid reactive substances (TBARS) level. Superoxide dismutase activity was significantly higher on days 9 ($P<0.05$) and 14 ($P<0.01$) in the treated group compared to the control rats. By contrast, lipid peroxidation, as TBARS value, reduced significantly on both experimental days in the ghrelin-exposed animals ($P<0.05$). Although, the mean activity of catalase and glutathione content was greater in the treated rats, however, the differences were not statistically significant. Slight changes occurred in glutathione peroxidase activity during the experimental period and there were no differences either on day 9 or on day 14 between groups. In conclusion, the results of the present investigation indicate for the first time the novel evidence of antioxidant properties of ghrelin in the rat ovary.

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1. Introduction

Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radical, are generated as byproducts of oxidative metabolism in mitochondria, can interact with biomolecules and damage various cellular components such as DNA, RNA, protein, and lipids [1–3]. Electron transport associated with steroidogenesis is an important site of oxygen radical generation in the testis and ovary [4]. Inadequate protection from ROS that are formed in sterioendogenically active granulosa and luteal cells could be a potential trigger for follicular atresia [4] and corpus luteum regression [5,6] in the rat ovary.

Ghrelin has been recently identified as an endogenous ligand for growth hormone secretagogue receptor (GHS-R) that regulates growth hormone secretion, increases appetite and contributes to energy homeostasis [7]. Recent studies strongly suggest the potential involvement of ghrelin in regulation of the reproductive axis. It has been detected in a large number of tissues and cell types, including hypothalamus, small intestine, pancreas, placenta, kidney, lung, pituitary, brain, ovary and testis [8–10]. In the ovary, expression of ghrelin has been demonstrated in steroidogenically active luteal and interstitial hilus cells. Likewise, expression of the functional ghrelin receptor has been reported in oocytes as well as follicular, luteal, surface epithelium and interstitial hilus cells in the rat ovary [11–13]. These observations highlight the plausibility for a role of ghrelin in the direct control of ovarian function and indicate that ovarian follicular and luteal cells are potential targets for systemic or locally produced ghrelin, because they express the functional type 1a of GHS-R.

In recent years, increasingly more evidence supports the hypothesis that ghrelin may be an antioxidant and anti-inflammatory agent. For example, it has proven that ghrelin prevents lipid peroxidation and reduction of antioxidant enzyme activities and glutathione level against pentyleneetetrazole-induced oxidative stress in the erythrocytes, liver and brain of rats [14]. Zwirska-Korczala et al. [15] demonstrated that ghrelin significantly increases the activity of antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), and that it decreases the concentration of malondialdehyde (MDA), an end product of lipid peroxidation, in preadipocyte cell culture. Likewise, Iseri et al. [16] showed that ghrelin treatment significantly increased GPx activities...
and reduced MDA levels in the alendronate-induced gastric tissue injury in rats. This same study found that ghrelin decreased formation of reactive oxygen species (ROS). Ghrelin has also been shown to inhibit vascular superoxide production and oxidative stress in hypertensive rats by inhibition of vascular NADPH oxidase [17], and to increase mRNA levels of SOD in trout phagocytic leukocytes [18]. Additional studies have echoed the proposal that ghrelin attenuates the oxidative stress responses [19]. In this sense, antioxidant properties of ghrelin were recently shown in our laboratory in the rat testis [20–22]. With regard to previous studies, we designed present investigation to determine probable antioxidant effects of ghrelin in the rat ovary. Therefore, the present study attempted to clarify the possible antioxidant properties of ghrelin using measurement of the activities of major antioxidant enzymes including SOD, GPx and CAT as well as glutathione (GSH) content and lipid peroxidation following chronic administration of ghrelin in the rat ovary.

2. Materials and methods

2.1. 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 enzyme activities were provided from Randox Laboratories Ltd. (Antrim, UK). Other chemicals were purchased from Sigma-Aldrich Company (St. Louis, MO, USA) unless otherwise indicated.

2.2. Animals

The experiment was carried out on twenty eight adult female Wistar rats with 10 weeks of age. The animals were obtained from Razi Research Institute in Karaj, Iran. The rats were housed (seven rats per cage) inanimal room under controlled lighting (14 h light: 10 h darkness, lights from 06:00 h) and temperature (21–24 °C) conditions and had free access to a pelleted food and tap water ad libitum. All of the experimental procedures were carried out between 09.00 and 11.00 am. All investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals. All animals were treated humanely and in compliance with the recommendations of Animal Care Committee for the Lorestan University of Medical Sciences (Khorram Abad, Iran).

2.3. Experimental design

The animals were randomly allocated to four groups (each containing 7 rats) as two control and treatment groups. In order to verify the hypothesis that ghrelin treatment might affect the ovarian antioxidative defense mechanism, a general protocol of subcutaneous (S.C) injection of ghrelin (2 nmol/100 µl saline) or 100 µl vehicle (physiological saline) to the control group was applied once a day for 14 consecutive days. The dose used in our in vivo setting, is in the physiological range of circulating ghrelin in the body. Because, it has been proved that exogenous administration of 1 nmol of ghrelin is able to induce a significant elevation (2.4- to 2.6-fold increase) in serum levels of total ghrelin 1 h after injection [8], whose magnitude is in the range of that induced by fasting [23]. Also the period of ghrelin treatment in our study, was in accordance on the fact that the mean duration of estrous cycle in female rat is approximately 4.4 days [24]. Thus, the animals treated for over three estrous cycles. The animals were injected under conscious conditions after careful handling to avoid any stressful influence. The rats from both groups were killed upon diethyl ether anesthesia (May & Baker Ltd, Dagenham, England) on 9th (n = 14) and 14th (n = 14) days of the treatment by decapitation.

2.4. Sampling and tissue preparation for enzyme assay

Immediately after rat killing on days 9 and 14, both ovaries were taken and carefully dissected from the surrounding fat and tissue and processed immediately after killing. In order to perform enzyme and lipid peroxidation assays, fresh (unfrozen) ovaries were rapidly homogenized manually in cold phosphate buffer (pH 7.4) and debris removed by centrifugation at 3500 g for 10 min (Rotofax 32 A, Hettich, Tuttingen, Germany). The upper clear supernatants were recovered and stored at −70 °C for later enzyme and protein assays.

2.4.1. GPx assay

The activity of GPx was evaluated with GPx detection kit according to the manufacturer’s instructions. GPx catalyzes the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+ . The decrease in absorbance at 340 nm against blank was measured spectrophotometrically (Pharmacia Biotech, Ultraspec 3000, UV, Cambridge, UK). One unit (U) of GPx activity was defined as the amount of enzyme that converts 1 µmol of NADPH to NADP+ per minute. The GPx activity was expressed as unit per milligram of tissue protein (U/mg protein).

2.4.2. SOD assay

Total SOD activity was evaluated with SOD detection kit according to the manufacturer’s instructions. The role of SOD is to accelerate the dismutation of the toxic superoxide (O2·−) produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iiodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) 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 protein (U/mg protein).

2.4.3. CAT assay

Tissue catalase activity was assayed using the method described by Claiborne [25]. The reaction mixture (1 ml) consisted of 50 mM potassium phosphate (pH 7.0), 19 mM H2O2, and a 20–50 µl sample. The reaction was initiated by the addition of H2O2 and absorbance changes were measured at 240 nm (25 °C) for 30 s. The molar extinction coefficient for H2O2 is 43.6 M−1 cm−1. The CAT activity was expressed as the unit that is defined as µmol of H2O2 consumed per min per gram of wet tissue.

2.4.4. Total GSH content

Total GSH was estimated by the method described by Sedlak and Lindsay [26]. Briefly, 5% tissue homogenates were prepared in 20 mM EDTA, pH 4.7, and 100 µl of the homogenate or pure GSH was added to 0.2 M Tris–EDTA (1.0 ml, pH 8.2) buffer (Fluka, Switzerland) and 20 mM EDTA, pH 4.7 (0.9 ml) followed by 20 µl of Ellman’s reagent (10 mmol/1 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 homogenates, 100 µl of distilled water was added. 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.

2.4.5. Measurement of lipid peroxidation

The level of lipid peroxidation was indicated by the content of TBARS in the ovary. Tissue TBARS was determined by following the production of thiobarbituric acid reactive substances as described by
Subbarao et al. [27]. In short, 40 µl of homogenate was added to 40 µl of 0.9% NaCl and 40 µl of deionized H₂O, resulting in a total reaction volume of 120 µl. The reaction was incubated at 37 °C for 20 min and stopped by the addition of 600 µl of cold 0.8 M hydrochloric acid (HCl), containing 12.5% trichloroacetic acid (TCA). Following the addition of 780 µl of 1% TBA, the reaction was boiled for 20 min and then cooled at 4 °C for 1 h. 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 × 10⁵. 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).

2.4.6. Protein measurement

Protein content of tissue homogenates was determined by a colorimetric method of Lowry using bovine serum albumin as standard [28].

2.5. Statistical analysis

All values are given as mean ± SEM. The data were subjected to Levene's test for homogeneity of variances. The activities of SOD, GPx and CAT as well as GSH content and TBARS level on days 9 and 14 between the control and treated groups were compared by independent sample t-test [29]. Significant level was set at P < 0.05.

3. Results

The mean values of GPx, SOD and CAT activities as well as GSH content in the rat ovarian tissue on days 9 and 14 are presented in Table 1 and 2. Clearly, chronic administration of ghrelin significantly increased the activity of SOD on both experimental killing days in the ghrelin-exposed rats compared to the control group (P < 0.05). So that, the mean activity of this enzyme was much pronounced (over than three-fold increase in its activity) on days 9 and 14 in the injected animals compared to the control rats. Although, ghrelin could enhance the mean activity of CAT approximately 1.5-fold in the treated animals, in which ghrelin caused up to 25% increment in GSH content on day 9 of treatment. However, the treated animals, in which ghrelin caused up to 25% increment in GSH content and TBARS level on days 9 and 14 (P < 0.05) for homogeneity of variances. The activities of SOD, GPx and CAT as well as GSH content (despite of their increased values in the treated rats) did not exhibit any significant changes between groups. By contrast, ghrelin could considerably influence lipid peroxidation products in the ovarian tissue, in which, the TBARS level was significantly lower in the treated animals.

The intensive metabolism of granulosa cells and the high numbers of macrophages and neutrophilic granulocytes in the follicular wall at ovulation may point to active generation of ROS [30]. Margolin et al. [31] observed that ROS are involved in the loss of sensitivity of granulosa cells to gonadotropic hormones and in the loss of steroidogenic function, both of which are characteristics of follicular atresia. Normally, cells possess a well-developed biochemical defense system, comprising low-molecular weight free radical scavengers, i.e. GSH, vitamin C, vitamin E, and complex enzymes, including GPx, SOD and CAT [32]. Inhibiting the ability of a cell to scavenge or detoxify ROS is another way by which oxidative stress can induce apoptosis.

There are some mechanisms for ghrelin action in the ovary. Expression of ghrelin mRNA and its cognate receptor has been demonstrated in rat and human ovaries. Ovarian expression of GHS-R1a shows a wide pattern of tissue distribution, with detectable specific signal in oocytes as well as somatic follicular cells, luteal cells and interstitial hilus cells. Of particular note, follicular GHS-R1a expression parallels follicle development with stronger immunostaining in granulosa and theca layers of healthy antral follicles [13]. Moreover, persistent expression of ghrelin gene was demonstrated in rat ovary throughout the estrous cycle, with the lowest level in the proestrus and peak expression on the first day of the diestrous. Such a cyclic profile of expression, with peak level in the luteal stage, is highly suggestive of predominant expression of ghrelin in the corpora lutea of the current cycle [11]. The presence of both components (ligand and receptor) of the ghrelin signaling system within the ovary

Table 1
Mean ± SEM of ovarian antioxidative enzyme activities (SOD, GPx and CAT) as well as GSH content on day 9 of treatment.

<table>
<thead>
<tr>
<th></th>
<th>SOD (U/mg protein)</th>
<th>GPx (U/mg protein)</th>
<th>CAT (U/mg protein)</th>
<th>GSH (µmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=7)</td>
<td>30.46 ± 9.24a</td>
<td>8.83 ± 1.73a</td>
<td>13.94 ± 4.94a</td>
<td>0.13 ± 0.02a</td>
</tr>
<tr>
<td>Treatment (n=7)</td>
<td>112.89 ± 27.87b</td>
<td>9.19 ± 2.98b</td>
<td>25.24 ± 10.63b</td>
<td>0.13 ± 0.02a</td>
</tr>
</tbody>
</table>

*Means in the same columns with different superscripts significantly differ (P < 0.05).

Table 2
Mean ± SEM of ovarian antioxidative enzyme activities (SOD, GPx and CAT) as well as GSH content on day 14 of treatment.

<table>
<thead>
<tr>
<th></th>
<th>SOD (U/mg protein)</th>
<th>GPx (U/mg protein)</th>
<th>CAT (U/mg protein)</th>
<th>GSH (µmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=7)</td>
<td>33.78 ± 7.53a</td>
<td>7.45 ± 0.70a</td>
<td>27.74 ± 5.38a</td>
<td>0.19 ± 0.02a</td>
</tr>
<tr>
<td>Treatment (n=7)</td>
<td>107.99 ± 17.92b</td>
<td>7.53 ± 0.64a</td>
<td>36.41 ± 3.42b</td>
<td>0.24 ± 0.01a</td>
</tr>
</tbody>
</table>

*Means in the same columns with different superscripts significantly differ (P < 0.05).
opens up the possibility of a potential regulatory role of this novel molecule in ovarian function under physiological condition. Furthermore, ghrelin can influence ovarian cells directly, through changes in release of ovarian hormones. For instance, 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 estrogen biosynthesis [33,34]. Estrogen is one of the non enzymatic antioxidant in the ovary [35].

The presence of different antioxidant defense systems is well documented in the rat ovary. Corpus luteum has an antioxidant enzyme to scavenge ROS: Cu, Zn-SOD. Decrease in intracellular SOD activity inhibits progesterone production by rat luteal cells and results in the loss of luteal function, which may be mediated by ROS [6]. Moreover, the role of GPx in maintaining low concentrations of hydroperoxides inside the follicle has been suggested, in which, the mean GPx activity in the follicular fluid was found to be ~70% of its serum activity [36]. Jozwik et al. [37] showed that the intensity of peroxidation in the Graafian follicle is much lower than that in serum. This gradient is the result of the lower rate of initiation of peroxidation in the follicular fluid, suggestive of the presence of efficient antioxidant defense systems in the direct milieu of the oocyte such as GSH [4] and GPx [36].

It has been suggested that accumulation of ROS and a decrease in SOD levels are involved in apoptotic cell death, whereas antioxidants including SOD can inhibit apoptosis [38]. In our results, ghrelin considerably increased SOD activity, as a key antioxidant enzyme against oxidative damage, in the rat ovarian tissue more than three folds compared to the control group. Enhanced level of this antioxidant enzyme activity in the ovary suggested scavenging of free radicals from the ovarian tissue following exposure of ghrelin and prevention of destructive effect of oxidative stress in the ovaries. SOD rapidly converts superoxide anion (O2−) to less dangerous hydrogen peroxide (H2O2) and then GPx and CAT can decompose H2O2 to water. Although, H2O2 is not a particularly reactive product, but it may be reduced to the highly reactive metabolites hydroxyl radicals (OH·) or single oxygen [39].

Recently, it has been demonstrated that growth hormone (GH) stimulated both ghrelin synthesis and secretion in the ovarian follicles. Also a significant increase in local ovarian GH secretion was observed under the influence of ghrelin. The stimulatory action of GH but not IGF-1 on ghrelin synthesis and secretion by ovarian cells suggests a possibility of existence of a feedback loop of GH and ghrelin but not IGF-1 and ghrelin in the ovary. On the other hand, the presence of GHS-R1a in the ovary, together with the fact that ghrelin increases local GH secretion in the follicles whereas GH increased local ghrelin synthesis and secretion, leaves little doubt about the existence of local GH–ghrelin axis in the ovary [33]. Furthermore, it has been shown that ghrelin acts on several key steroidogenic enzymes in rat testis [40]. Recent study evidenced that androgens are independent modulators of ghrelin levels in women, thus confirming an interaction between ghrelin and sex steroid synthesis [41]. On the other hand, it has been shown that GH increased FSH-stimulated granulosa cell differentiation and aromatase activity in rats and estradiol production by human ovaries. Similarly, a direct gonadotrophic effect of GH on estradiol production by human granulosa cells has been reported [42]. Estrogen may also 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 [43].

ROS cause membrane damage of luteal cells [44,45], inhibit the cholesterol transport to mitochondria in luteal cells [46] and therefore reduce progesterone production. As indicated in our data, lipid peroxidation, which functions as a marker of oxidative injury of cellular membranes [47,48] significantly decreased following daily administration of ghrelin for 14 days. The concentration of MDA is a direct evidence of toxic processes caused by free radicals [49]. Therefore, it can be concluded that ghrelin preserves the ovarian cell membranes against oxidative stresses and lipid peroxidation. These findings support and are in agreement with our new investigation, in which we proved that chronic administration of ghrelin increases functional membrane integrity of rat spermatozoa [20]. Likewise, the antioxidant properties of ghrelin are consistent with our another study, in which we have shown that ghrelin enhances viability of rat spermatozoa during incubation at 37 °C up to 5 h, because of its antioxidant characteristics [21].

The increase in SOD activity in our investigation correlates well with the decrease in lipid peroxidation in the ovarian tissue. SOD shifts highly reactive O2 to H2O2 and thus prevents the ovarian cell membrane damage caused by this highly toxic anion. This finding indicates that ghrelin reduces superoxide radical production and therefore decreases formation of lipid peroxidation products. This is further supported by the fact that we detected lower TBARS content in the rat ovary and therefore, the reduction in TBARS concentration in this work can be justified. Similar results were also found in our recent study, in which ghrelin increased GPx activity and reduced lipid peroxidation in the rat testis following 10 days of administration, however failed to change SOD or CAT activity [22].

H2O2 was found to be a pluripotent inhibitor of progesterone synthesis in cultured granulosa luteal cells in humans [50]. In the rat, the luteolytic action of PGE2α was associated with H2O2 and lipid peroxide generation [5]. CAT catalyzes the decomposition of H2O2 to produce water and molecular oxygen, and plays a major role in protecting cell against oxidative damage [51]. Decrease in CAT activity may compromise the overall antioxidant enzyme defense system. However, it is well known that GPx per se is not an efficient H2O2-decomposer and high levels of H2O2 occur in GPx-sufficient, but CAT-depleted cells [51,52]. Therefore, the compromised CAT activities may significantly impair the capacity of antioxidant enzyme defense mechanism [53]. Although the mean activity of CAT in our study was approximately 1.5-fold higher in the ghrelin-treated group, however, the difference was not statistically significant. Possibly, prolonged treatment by ghrelin or higher doses is needed to induce greater activity of CAT in the ovary. Further researches are necessary to confirm this hypothesis. On the other hand, as indicated in the tables, GPx seems to be less affected by ghrelin and this hormone could not markedly influence ovarian GPx activity, because of its slight alterations throughout the experimental period. Possibly, one of the reasons for unaffected GPx level may be due to the presence of non enzymatic antioxidant in the follicular fluid of the ovary other than GPx which significantly reduces lipid hydroperoxide concentrations in the fluid of preovulatory follicles [54]. A factor responsible for this effective antioxidant action may be estrogens [35].

Recently, in vivo and in vitro antioxidant activity of ghrelin has been indicated in gastric ischemic injury in the rat by El Eter et al. [55]. They showed that protective effect of exogenous ghrelin is mediated by its antioxidant activity in vivo by decrease in the tissue level of TBARS, as an indicator of lipid peroxidation. Moreover, ghrelin could resume the depleted GSH level to the normal value in that study, suggesting an in vivo antioxidant activity of this peptide. GSH is a physiological reductant that maintains the intracellular environment in a mildly reductive condition [56]. These data suggest that ghrelin directs the redox reactions toward the reduction state thus reducing the reactivity of oxygen free radicals with other molecules. GSH is the most abundant tripeptide thiol antioxidants as a direct ROS scavenger and the substrate of GSH-related detoxifying and antioxidant enzymes [48]. Several studies have indicated a relationship between intracellular glutathione depletion and apoptosis. It has been suggested that oxidative stress induces apoptosis in preovulatory follicles and that the antipoptotic effect of FSH is mediated in part by stimulation of follicular GSH synthesis and suppression of ROS production [4]. Beaver and Waring [57] showed that a decrease in
intracellular GSH or an increase in glutathione disulphide (GSSG, oxidized glutathione), or perhaps a change in the ratio of GSH to GSSG, constitutes a trigger for apoptosis. The abnormalities in follicular glutathione systems can decrease the ability of follicles to respond to oxidative stress or toxicant exposures, causing increased follicular apoptosis and even ovarian failure [4]. These observations highlight the importance of GSH in the female reproductive oxidative stress control. In the present study, repeated chronic injection of ghrelin could enhance by 25% of GSH content of ovarian tissue in the treated rats on day 14. Although this increment found to be not significant, however, like to CAT it is possible that, longer time or higher doses administration can promote GSH content significantly.

In conclusion, the results of the present study demonstrated the novel evidence of antioxidant properties of ghrelin in the rat ovary. This finding confirms the recent reports concerning the antioxidative properties of ghrelin in the other tissues [14–17].

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