Ghrelin enhances viability of rat spermatozoa during incubation at 37°C

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Summary

Antioxidant properties of ghrelin have been demonstrated in recent studies. In the present study, the effects of chronic administration of ghrelin on the motility and plasma membrane integrity of rat spermatozoa during incubation at 37ºC were investigated. Thirty 45-day-old male Wistar rats were divided into control and treatment groups. Rats in the treatment group were daily injected subcutaneously with 1 nmol of ghrelin for 10 consecutive days and the control rats received normal saline. Sperm was collected after killing of rats on days 5, 15 and 40 after the last injection, and sperm characteristics were examined at 0, 3 and 5 h after incubation at 37ºC. Mass motility and forward progressive movement of spermatozoa were significantly higher in ghrelin-treated animals at 3 and 5 h of incubation on day 5 ($P<0.05$). After 3 h of incubation on day 15, only mass motility was greater than that of the control group. Plasma membrane integrity was assessed by hypoosmotic swelling (HOS) "water test". The mean value of HOS reacted spermatozoa was higher in the treatment group on days 5 and 15 during 0, 3 and 5 h of incubation ($P<0.05$). However, the percentage of HOS-positive spermatozoa was not significantly different on day 40 between groups. There was a high correlation at 3 and 5 h of day 5 between the forward progressive movement ($r = 0.92$ and 0.94, $P<0.0001$) as well as overall sperm motility ($r = 0.78$ and 0.81, $P<0.01$) with HOS test in the ghrelin-treated animals. These results can be attributed to the antioxidative effects of ghrelin on the rat sperm especially on its plasma membrane which probably protects the sperm plasma membrane against oxidative damage during incubation and causes subsequent significant increase in the HOS test results. This may result in higher sperm motility index during 5 h of incubation.

Key words: Ghrelin, Spermatozoa, Antioxidant properties, Rat, HOS test

Introduction

Ghrelin has been recently identified as an endogenous ligand for growth hormone secretagogue receptor that regulates growth hormone secretion, increases appetite and contributes to energy homeostasis (Kojima and Kanagawa, 2005). There is a close association between the systems governing energy homeostasis and reproductive function (Fernandez-Fernandez *et al.*, 2005). Recent studies strongly suggest the potential involvement of ghrelin in regulation of the reproductive axis. Ghrelin was shown to suppress luteinizing hormone (LH) secretion *in vivo* and to decrease LH responsiveness to GnRH *in vitro*. Moreover, ghrelin inhibited the testicular testosterone secretion *in vitro* (Tena-Sempere *et al.*, 2002; Barreiro and Tena-Sempere, 2004; Fernandez-Fernandez *et al.*, 2005; Fernandez-Fernandez *et al.*, 2006).

Recently, it was proposed that ghrelin may act as an antioxidant and/or anti-inflammatory agent. Zwirska-Korczala *et al.* (2007) demonstrated that ghrelin significantly increased the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and decreased the level of malondialdehyde (MDA), an end product of lipid peroxidation, in preadipocyte cell cultures. Likewise, Iseri *et al*. (2005) showed that ghrelin significantly increased GPx activity and reduced MDA levels in the alendronate-induced gastric tissue injury in rats. Also they found that ghrelin decreased formation of reactive oxygen species (ROS). Ghrelin also inhibited vascular superoxide production and oxidative stress in hypertensive rats by inhibition of vascular NADPH oxidase (Kawczynska-Drozdz *et al*., 2006) and increased mRNA levels of SOD in trout phagocytic leukocytes (Yada *et al*., 2006).

Oxidative damage of spermatozoa is a potential cause of decline in sperm motility and fertility upon storage of fresh liquid semen (Ball *et al*., 2001). The survival of sperm in the seminal plasma alone is limited to a few hours. To maintain sperm for longer periods, dilution with a protective solution containing antioxidant components is necessary for sperm membrane integrity and oxidative stress prevention (Maxwell and Stojanov, 1996; Sanchez-Partida *et al*., 1997; Uperti *et al*., 1998; Sarlos *et al*., 2002).

On the other hand, it is believed that ghrelin is one of the endogenous antioxidant that attenuates the oxidative stress response (Dong and Kaunitz, 2006). However, to date there is no literature pertaining to the probable antioxidative effects of ghrelin on the sperm viability and membrane integrity. Therefore, the objective of the present investigation was to evaluate the possible antioxidant properties of ghrelin on the motility and membrane integrity of rat spermatozoa during incubation at 37ºC.

Materials and Methods

Animals and drugs

Wistar male rats were purchased from the Razi Institute in Iran. The day the litters were born was considered day 1 of age. The rats were housed (5 per cage) in animal room under controlled lighting (14 h light: 10 h darkness, lights from 06:00 h) and temperature (21-24ºC) conditions and had free access to a pelleted food and tap water. Rat ghrelin was purchased from Tocris Cookson Ltd (Bristol, UK). All animals were treated humanely and in compliance with the recommendations of Animal Care Committee for the Lorestan University of Medical Sciences (Khorramabad, Iran).

Experimental procedure

Thirty pubertal-early adult (45-day-old) male rats were used in the study. This period was selected on the basis of previous study of the normal timing of puberty in the male rat (Ojeda and Urbanski, 1994). They were divided into control (n = 15) and experimental (n = 15) groups. The experimental group was subcutaneously injected with ghrelin (1 nmol/100 µl in normal saline) and the control group was administered with 100 µl saline for 10 consecutive days. This dose of ghrelin used in our *in vivo* setting, is close to that induced by fasting; because, exogenous administration of 1 nmol of ghrelin was able to induce a significant elevation (2.4- to 2.6-fold increase) in serum levels of total ghrelin 1 h after injection (Fernandez-Fernandez *et al*., 2005), whose magnitude is within the range induced by fasting (Wren *et al*., 2001). The animals were injected under conscious conditions after careful handling to avoid any stressful influence. The rats were killed by decapitation on days 5 (n = 5), 15 (n = 5) and 40 (n = 5) after the last injection of ghrelin for sperm collection and evaluation.

Sperm collection and evaluation

Immediately after killing, the right epididymis was removed and trimmed of fat. Spermatozoa were obtained and prepared by the method of Kato *et al*. (2002). Briefly, cauda epididymis was minced in synthetic human tubal fluid (HTF) medium (Quinn *et al*., 1985; Liu *et al*., 2007) and incubated at 37ºC for 30 min to allow dispersion of spermatozoa. The medium was filtered through a 0.22 µm filter and equilibrated with 5% CO_2 in air at 37ºC before use. Concentration of spermatozoa was determined by hemocytometer and aliquots of the sperm suspension (<50 µl, adjusted to about 1×10^6 cells/ml) were transferred to 2 ml of fresh medium. Samples were incubated in a 37ºC incubator for 3 and 5 h. This is because rat spermatozoa undergo capacitation at least 3 h of incubation, and 5 h of incubation is required in IVF for successful fertilization (Kato *et al*., 2002).

The obtained spermatozoa from the injected and control rats were assessed for total sperm motion (TSM), forward progressive motility (FPM) and plasma membrane integrity (PMI) immediately after collection (0 h) and after incubation for 3 and 5 h. Total sperm motility (cells showing any kind of movement) and FPM percentage (the motile spermatozoa showing progressive movement) were assessed according to Sonmez *et al*. (2005). The fluid obtained from cauda epididymis was diluted to 2 ml with PBS and an aliquot of this solution was placed on the microscope slide covered with a coverslip and examined visually under a phase-contrast microscope (Leica, USA) at the magnification of 400. For motility test, the mean of 200 spermatozoa from four different microscopic fields was used as the final motility score. Samples for motility evaluation were kept at 37ºC.

Evaluation of PMI was determined by hypoosmotic swelling (HOS) water test (Sliwa and Macura, 2005). Briefly, 10 µl of sperm suspension was added into 0.4 ml of distilled water and incubated for 5 min at 37ºC. The swelling reaction was measured by counting of sperm with curled/swollen tail using a phase-contrast microscope at ×400. In all examinations, at least 200 spermatozoa were counted.

Statistical analysis

Results were analyzed by SPSS software. At first, all data were assessed for normality and followed by Levene's test for homogeneity of variances. The effects of ghrelin on TSM, FPM and PMI were compared by independent sample t-test. The correlation between TSM and FPM with HOS test percentage at different hours of incubation was determined by the Pearson's correlation test (Petrie and Watson, 1999). Data are presented as the mean ± SEM. The level of significant was set at $p<0.05$.

Results

Although, immediately after sperm collection (0 h), the means of TSM or FPM percentage were higher in ghrelin-treated rats, they did not significantly differ between the control and treatment groups ($P>0.05$) on days 5 and 15 (Table 1). There was a high correlation ($r = 0.79$) between the FPM and percentage of HOS-reacted spermatozoa in the treated rats ($P<0.0001$). A positive correlation ($r = 0.52$) was also found between the TSM and HOS test in this group at 0 h ($P<0.01$).

After 3 h of incubation, both TSM (59.4% *vs*. 53.0%) and FPM (52.0% *vs*. 46.7%) were greater on day 5 in the treated animals ($P<0.05$). While on day 15 only TSM was significantly higher when compared to the control animals (59.4% *vs*. 52.7%, $P<0.05$). However, TSM and FPM did not display significant changes at 3 h on day 40 (Table 2).

After 5 h of incubation, the percentage of TSM and FPM was higher than those of the control group on day 5 ($P<0.05$), however, sperm motility was similar

Table 1: Mean ± SEM percentage of sperm parameters in the control and ghrelin-treated rats immediately after collection

0 h (before incubation)	Day 5		Day 15		Day 40	
	Control	Treatment	Control	Treatment	Control	Treatment
Total sperm motility (%)	65.7±2.0	70.4±2.1	64.7±1.8	69.2±1.7	66.2±2.8	65.2±2.1
Forward progressive movement (%)	57.5±1.7	62.4±1.8	57.0±1.5	61.2±2.2	57.2±2.3	56.8±1.9
HOS-positive (%)	68.2±2.0	82.2±0.6***	67.5±1.3	76.8±1.2**	69.0±1.2	67.2±1.8

**Significant at P<0.01 and ***Significant at P<0.001

Table 2: Mean ± SEM percentage of sperm parameters in the control and ghrelin-treated rats at 3 h after incubation

After 3 h of incubation	Day 5		Day 15		Day 40	
	Control	Treatment	Control	Treatment	Control	Treatment
Total sperm motility (%)	53.0±2.7	59.4±1.0*	52.7±2.5	59.4±1.1*	53.5±2.5	54.0±1.4
Forward progressive movement (%)	46.7±1.2	52.0±1.3*	45.5±0.9	47.4±1.6	46.2±0.7	46.8±1.5
HOS-positive (%)	59.5±1.5	66.2±1.5*	57.2±1.3	63.8±1.8*	57.7±1.4	56.8±1.2

*Significant at P<0.05

Table 3: Mean ± SEM percentage of sperm parameters in the control and ghrelin-treated rats at 5 h after incubation

After 5 h of incubation	Day 5		Day 15		Day 40	
	Control	Treatment	Control	Treatment	Control	Treatment
Total sperm motility (%)	43.2±1.7	48.2±1.0*	42.2±1.3	44.6±1.0	41.7±1.1	43.0±1.1
Forward progressive movement (%)	35.7±1.7	40.6±1.0*	36.5±1.8	38.6±1.1	36.2±1.3	36.8±0.6
HOS-positive (%)	48.2±0.8	55.0±1.4*	45.7±1.6	52.8±1.2*	45.2±1.4	44.0±1.1

*Significant at P<0.05

(P>0.05) between groups on days 15 and 40 (Table 3).

The percentage of HOS-positive spermatozoa was greater at 0 h particularly on day 5 as well as day 15 in the treated animals (P<0.01). The mean value on day 5 was higher than day 15 (82.2% *vs*. 76.8%, P<0.05) and day 15 was still significantly higher (76.8% *vs*. 67.2%, P<0.01) than day 40 in the treatment groups. There was a time-dependent decrease in HOS-positive spermatozoa in the injected rats at 0 h on day 5 through day 40 of the experiment, so that HOS values were identical to those observed in the control group on day 40 (P>0.05). A significant difference was seen in the HOS-reacted spermatozoa at 3 and 5 h of incubation on days 5 and 15. Overall, the HOS test was greater on days 5 and 15 during 0, 3 and 5 h of incubation in the treated animals in comparison with the control group. However, on day 40 and at all incubation times, the changes in HOS percentage were not significantly different between groups.

At hours 3 and 5 of day 5, a significant correlation was seen between TSM and HOS test (r = 0.78 and 0.81, respectively) in the injected rats (P<0.01). The highest correlation was found between FPM and HOS test at hours 3 and 5 of day 5 (r = 0.92 and 0.94, respectively) in the treated group (P<0.0001).

There was a significant correlation (r = 0.87) between FPM and HOS test (P<0.05) at hours 3 and 5 on day 5 in the control group, however, no significant correlation was found between TSM and HOS test at different hours of the experimental days in this group.

Discussion

The present study demonstrated the novel evidence for antioxidant properties of ghrelin on the rat spermatozoa during incubation at 37ºC. The percentage of HOS-reacted spermatozoa at 0, 3 and 5 h of days 5 and 15 as well as motility rate at 3 and 5 h of day 5 were significantly higher in the ghrelin-treated animals.

Oxidative stress is one of the factors associated with decline in fertility during semen storage. The sperm plasma membrane contains a high amount of unsaturated fatty acids and therefore is particularly susceptible to peroxidative damages with subsequent loss of membrane integrity, impaired cell function and decreased motility of spermatozoa (Aurich *et al*., 1997; Ball *et al*., 2001). Efforts for using of antioxidants supplementation are in order to decrease this peroxidation process.

A high correlation (r = 0.90) was reported by Jeyendran *et al*. (1984) between the percentage of sperm in semen sample that underwent swelling and the percentage of zona-free hamster oocytes that were penetrated by sperm from the same semen samples. Likewise, in human, a positive correlation between the percentage of swollen sperm and that of motile sperm was seen (Chan *et al*., 1985). Such a positive correlation was found in our investigation between the FPM and TSM with the HOS test percentage especially on day 5 in the ghrelin-treated animals. In addition, the results of our study showed that ghrelin ameliorated the PMI of spermatozoa at 0, 3 and 5 h after incubation on days 5 and 15 of sperm evaluation and this enhancement was possibly due to the antioxidative effects of ghrelin which caused higher motility rate of spermatozoa examined at these times. This may be a reason for greater sperm motility in the treated animals simultaneous with the high HOS percentage. This finding shows the suppression of oxidative process by ghrelin during incubation of sperm for IVF or other sperm manipulation techniques and improving sperm kinematic parameters.

There are several studies pertaining to the effects of vitamin E and ascorbic acid supplementation on rat sperm quality during toxicant or oxidative damage. For example, exogenous supplementation with ascorbic acid decreased lipid peroxidation and increased sperm concentration and plasma testosterone level in male rats and supplementation with vitamin E and/or vitamin C restored reduced glutathione (GSH) level and activity of SOD, CAT, GPx and glutathione reductase (GR) to normal range and decreased the levels of ROS and lipid peroxidation (Sen Gupta *et al*., 2004; Sonmez *et al*., 2005). Glutathione, the main intracellular non-protein sulfhydryl, plays an important role in the maintenance of cellular proteins and lipids in their functional state and provides major protection by participating in the cellular defense systems against oxidative damage (Ross, 1988).

It is possible that ghrelin may function as an intracellular antioxidant scavenging for free radical oxygen and lipid peroxidases by converting them to non-reactive forms; thus maintaining the integrity of membrane phospholipids (Smith and Akinbamijo, 2000). Because, it was shown that ghrelin decreases formation of ROS in alendronate-induced gastric injury (Sibila *et al*., 2003; Iseri *et al*., 2005). The ROS may cause lipid peroxidation of sperm cell membranes and consequently damage of midpiece and axonemal structure, malfunction of capacitation and acrosome reaction, loss of motility and may ultimately result in infertility (Tramer *et al*., 1998).

The present work demonstrated that ghrelin increased the PMI percentage of rat spermatozoa at all of hours and days of assessment except for day 40. Possibly, during 40 days namely after the last injection, ghrelin effects were attenuated or disappeared and it seems that this time is too long for PMI evaluation. This finding has also been proved in our new investigation (Kheradmand *et al*., 2009).

Potential harmful effects of ROS are eliminated by stimulation of antioxidant enzymes (Hu *et al*., 2005). Very recently, it was reported that ghrelin increased the activity of these enzymes including SOD, CAT, GPx and decreased the level of MDA in preadipocyte cell lines (Zwirska-Korczala *et al*., 2007). Bauche *et al*. (1994) observed differential distribution of these enzymes among rat testicular cells, showing that, the Sertoli cells had elevated SOD and GSH-dependent enzyme activities. Round spermatids were characterized by higher SOD activities and GSH content. Spermatozoa exhibited the same enzymatic systems but were devoid of GSH. Indeed, ghrelin may also increase the activity of these antioxidant enzymes during spermatogenic cycle within the rat testis and/or epididymis. Therefore, enhancement in the PMI of rat spermatozoa by exogenous ghrelin during incubation at 37ºC is likely related to increase of antioxidant enzyme activities. Certainly, measurement of these enzymes following ghrelin administration in the rat testes and epididymis will be helpful and needs further studies.

In conclusion, chronic administration of ghrelin, increased PMI and viability of rat spermatozoa immediately after sperm collection and during incubation at 37ºC until 5 h. This finding confirmed the recent reports concerning the antioxidant effects of ghrelin in other tissues (Iseri *et al*., 2005; Kawczynska-Drozdz *et al*., 2006; Zwirska-Korczala *et al*., 2007).

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