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GHRELIN AS A REGULATOR OF GONADAL FUNCTION IN ADULT MALE OFFSPRINGS EXPOSED TO CIGARETTE SMOKING IN UTERO

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Objective. This study was carried out to evaluate serum and testicular ghrelin levels and to find whether it is related to pituitary-gonadal axis in adult male offsprings exposed to cigarette smoking *in utero***.**

Methods. Adult male Sprague Dawley rats were divided in two groups: 1. 20 rats born to mothers exposed to cigarette smoking (CS) during pregnancy (CSE group); 2. 20 rats born to normal non exposed pregnant rats (control group). Ghrelin concentration was determined by ELISA in the sera and testicular tissue of adult offsprings and also in sera of their mothers. Serum and testicular testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were evaluated using chemiluminescence immunoassay in adult male rats. In male offspring also the body weight and testicular weight was measured.

Results. Significantly higher ghrelin levels were found in serum and testicular homogenates of CSE male rats and also in sera of their mothers when compared to controls ($p<0.001$). Moreover, in CSE male rats the body weight, testicular weight, serum and testicular testosterone as well as serum LH and FSH were significantly lower than these in controls ($p<0.001$). Significant positive correlation was found between serum and testicular ghrelin in CSE group, while, in contrast, the correlations between serum ghrelin level and the other studied parameters in the same group were significantly negative.

Conclusion. The data obtained supported the view that ghrelin operates as a modifier of pituitary-gonadal axis. Hyperghrelinemia as a result of maternal smoking during pregnancy may contribute to the suppression of male reproductive function. Further studies are needed to clarify the role of ghrelin after quitting smoking.

Keywords: Ghrelin – Testosterone – LH – FSH – Maternal smoking – Pituitary gonadal axis.

Maternal smoking during pregnancy is a major health problem for the male offspring, since it has many consequences on the fetus and later on the adult son (Knopik 2009). Smoking, as an anorexic agent, produces the reduction in food intake and increases the metabolic rate thus leading to lowering of body weight (Kokkinos et al. 2007). This adverse energy balance in smoking could result in growth impairment of the offspring in addition to dysregulation of certain endocrine and gonadal functions (Gaworski et al. 2004; Ramlau-Hansen et al. 2008).

Proper gonadal function relies on a complex regulatory network of systemic (endocrine) and locally produced (paracrine and autocrine) signals. A number of factors involved in the control of energy balance and metabolism have been proven as putative modulators of gonadal axis (Tena-Sempere et al. 2005). This involves the concerted actions of hormonal signals with key

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roles in the metabolism such as leptin and recently also ghrelin (Sirotkin et al. 2008).

Ghrelin, a 28–amino acid peptide has been recently identified as the endogenous ligand of growth hormone secretagogue receptor (GHS-R). This peptide is primarily expressed in the stomach and hypothalamus with the ability to stimulate growth hormone (GH) release (Vander Lely et al. 2004; Kojima and Kangawa 2005). The ghrelin gene is expressed in stomach, small intestine, brain, pituitary gland, salivary gland, adrenal gland and gonads, with maximum expression occurring in the stomach (Ghelardoni et al. 2006)

Interestingly, besides its role in the control of GH release, ghrelin likely from a stomach source has been recently emerged as an orexienic food intake controlling signal through its action upon the hypothalamic centers and the release of neuropeptide Y as well as agouti-related peptide (Tena-Sempere et al. 2002).

Despite the fact that most of the biological actions of ghrelin are carried out centrally, additional peripheral actions of ghrelin have recently emerged (Gaytan et al. 2004). In this sense, novel expression of ghrelin in non central tissue was reported (Ishikawa et al. 2007). Fuglsang et al. (2007) reported that ghrelin and its receptors are found in reproductive organs and placenta indicating its role in reproduction. The pregnancy related time course of ghrelin expression observed in placenta together with the recently reported presence of ghrelin in full term neonates cord samples suggest that this peptide might influence fetal growth during intrauterine development. (Chanoine et al. 2002).

The testis is a complex organ in which different cell types interplay to ensure male fertility under the control of an array of extragonadal and intragonadal hormones and growth factors (Barreiro et al. 2002). Within the testis, expression of ghrelin has been demonstrated in mature Leydig cells of rat and human. In addition, expression of the functional ghrelin receptor, the GHS-R type 1a, has been shown in Sertoli and Leydig cells (Kheramand et al. 2009). Moreover, the expression of GHS-R 1a in the seminiferous tubules strongly suggests that its epithelium might be a target for ghrelin action.

Therefore, it could be suggested that ghrelin may play a role in gonadal function. The purpose of this study was to evaluate serum and testicular ghrelin levels and to find whether it is related to pituitary-gonadal axis in male rats exposed to cigarette smoking *in utero*.

Materials and Methods

The local ethical committee approved the animal experimental protocol. Forty adult male Sprague Dawley rats aged about 3–4 months and weighing 150–250 g were obtained from the animal house of Medical Research Institute, Alexandria University, Egypt. Two groups of twenty adult male rats were used, one of them being born to mothers exposed to cigarette smoking during the whole period of pregnancy (cigarette smoke exposed - CSE group) and twenty rats born to normal pregnant non exposed mothers and served as controls (control group).

Cigarette smoking (CS) exposure. Five groups of four pregnant rats were placed in smoking chamber (closed box of 20 cm length, 40 cm breadth and 25 cm width. This box was airtight, having holes in two opposite sides and was covered by a glass lid through which the rats could be observed. A cigarette was fixed into a hole where the lit end was introduced into the box and the other end was fixed in a pump. Thus, by suction the cigarette smoke is introduced into the box. The type of cigarette used was a filter tipped containing 1 mg of nicotine/cigarette. Each four rats were exposed to the smoke of two cigarettes twice daily starting from the first day of pregnancy until the delivery. The other group of normal pregnant rats (non exposed) were transferred carefully from their ordinary cages to another chamber which was similar to the smoking chamber but without cigarette inhalation to be in the same circumstances as the cigarette smoking exposed group and for the same time, this was done twice daily. Then both the exposed and non exposed rats are returned back to their ordinary cages. The Rats were allowed free access to normal laboratory diet and water and offsprings were cared of by their mothers until weaned at day 21 postnatal. At the 20th day, blood samples were taken from the pregnant females of each group.

The adult male offspring aged 3–4 months were separated to be used in this study. Blood samples were obtained by cardiac puncture into glass tubes after an overnight fast and immediately centrifuged at 1500 rpm for 15 minutes. Serum was separated in 1.5 ml Eppendorph tubes, then stored frozen at -80°C until hormonal assay.

Preparation of testicular homogenate. Testes were dissected, cleaned from adhering matter, their weights were recorded and then homogenized in (0.015 M Na_2HPO_4, 0.15 M NaCl) buffer at pH 7.8 (10 % homogenate W/V) using Teflon automatic homogenizer. The supernatant obtained from the homogenate by ul-

Table 1

Body weight, testicular weight, serum and testicular testosterone and serum gonadotropins (LH and FSH) in control and adult male offsprings exposed to CS in utero

	Control (n = 20)	CS. Exposed (n = 20)
Body weight (gm)	193.5 ± 18.72	169.55 ± 17.65*
Testicular weight (gm)	3.29 ± 0.415	2.59 ± 0.31*
Serum testosterone (ng/ml)	3.63 ± 0.41	0.36 ± 0.44*
Testicular testosterone (ng/gm testis)	0.53 ± 0.23	0.03 ± 0.01*
Serum LH (MIU/ml)	3.02 ± 1.29	0.175 ± 0.08*
Serum FSH (MIU/ml)	4.96 ± 1.23	1.48 ± 0.76*

Data are expressed in mean ± SD.
* P < 0.001 when compared to control group.

tracentrifugation and filtration was kept at -70 °C until the hormonal assay .

Hormonal assays. Serum and testicular ghrelin was determined according to Portsman et al. (1992) employing a double antibody sandwich type of enzyme linked immunosorbant assay (ELISA) using kits purchased from Phoenix Pharmaceuticals (INC, USA). The measuring range was 0.12 – 1.26 ng/ml.

Serum and testicular testosterone was determined using electrochemiluminescence immunoassay (ECLIA). The testosterone ECLIA is based on a competitive test principle using a monoclonal testosterone specific antibody that was done according to WHEELER (1995) using the kits purchased from Roche Diagnostics (Mannheim, Germany) with a measuring range of 0.02–15 ng/ml.

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) were determined using ECLIA assay employing two different FSH and LH specific monoclonal antibodies and kits purchased from Roche Diagnostics (Mannheim, Germany) (BESTALL et al. 1987).

Statistical evaluation. All data were expressed as mean ± SD. Student t-test was used for the comparison of data between two studied groups. Pearson's correlation was done between serum ghrelin levels and the other studied parameters. Results were considered significant at p<0.05.

Results

In the group of adult male rats exposed to cigarette smoking (CS) *in utero*, the mean values of body weight, testicular weight, serum testosterone, testicular testosterone, serum LH and FSH were significantly lower than the control non exposed rats (P < 0.001). Table 1.

Pregnant dams exposed to CS throughout the whole period of pregnancy demonstrated significant increase in serum ghrelin when compared to non exposed pregnant rats (P < 0.001). (fig 1). As shown in (fig. 2), it is clearly evident that the *in utero* exposure to CS induced a significant increase in both serum and testicular ghrelin as compared to non exposed controls (P < 0.001).

Table (2) shows correlations between serum ghrelin concentrations and the other studied parameters in control and adult male offsprings exposed to CS *in utero.* In the non exposed control group, significant negative correlation was found only between serum ghrelin and LH (r=-0.57, P=0.008). However, in CS-exposed group, serum ghrelin concentrations were negatively correlated with body weight (r = -0.65, P = 0.002), testicular weight (r = -0.39, P = 0.09), serum testosterone

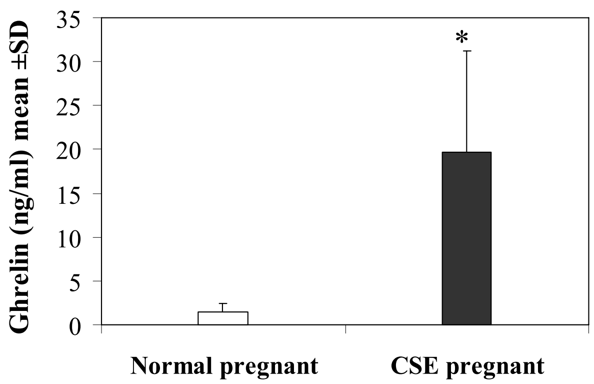

Fig. 1
Serum ghrelin levels (ng/ml) mean ± SD in normal pregnant and cigarette smoking exposed pregnant rats, *p<0.001 vs normal pregnant.

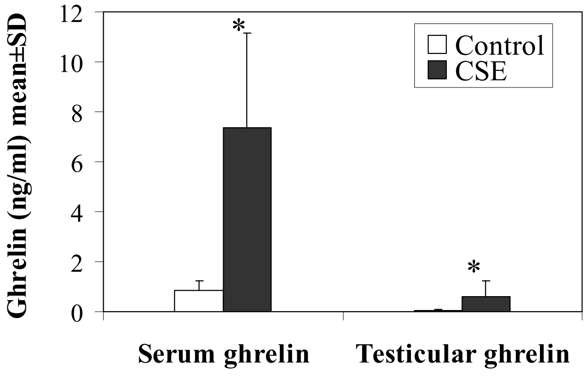

Figure 2
Serum and testicular ghrelin levels (ng/ml) mean ± SD in controls and adult male offsprings exposed to cigarette smoking in utero, * P<0.001 vs controls.

(r=-0.502, P = 0.02), testicular testosterone (r = - 0.456, P = 0.043), serum LH (r = -0.469, P = 0.037) and serum FSH (r = - 0.268, P = 0.25). while, serum ghrelin concentrations were significant positively correlated with testicular ghrelin (r = 0.55, p = 0.01).

Discussion

This study investigates the impact of maternal smoking during pregnancy on ghrelin levels and its relationship to pituitary gonadal axis in adult male offsprings exposed *in utero*. Given the known anorexic action of smoking, a decrease in ghrelin levels might be expected. However, the most interested finding in this work was the unexpected increase in ghrelin levels in sera of CS pregnant dams and their adult male offsprings as compared to normal rats.

During pregnancy, a number of physiological changes occur in maternal circulation to accommodate the growing fetus (Makino et al. 2002). Energy requirements are increased during pregnancy and the ingestive behaviour often changes to meet the metabolic demands (Shibata et al. 2004). It is possible that the decreased serum ghrelin levels obtained in the normal pregnant rats in this study may represent a physiological adaptation to the positive energy balance in pregnancy.

In addition, the increased ghrelin levels in sera of CS exposed dams and their adult male offsprings exposed *in utero* may be related to the role of this peptide in energy balance. Moreover, we could suggest that nicotine may exert its anorectic effect through modulation of ghrelin level and ghrelin signaling.

Makino et al. (2002) reported that plasma ghrelin levels are significantly decreased in normal pregnant women during the third trimester in comparison to non pregnant women. They suggested also that the higher ghrelin levels in the umbilical artery than in maternal blood may contribute to its production by fetal-placental unit.

Table 2

Correlation between serum ghrelin concentration and the studied parameters in controls and adult male offsprings exposed to CS in utero

	Control (n = 20)	CS. Exposed (n = 20)
Body weight (gm)	r = 0.35 P = 0.14	r = -0.65 p = 0.002
Testicular weight (gm)	r = 0.0251 p = 0.29	r = -0.39 p = 0.09
Testicular ghrelin (ng/gm testis)	r = 0.24 p = 0.29	r = 0.55 p = 0.01
Serum testosterone (ng/ml)	r = 0.14 p = 0.56	R = -0.502 p = 0.02
Testicular testosterone (ng/gm testis)	r = -0.39 p = 0.08	R = -0.456 p = 0.043
Serum LH (MIU/ml)	r = -0.57 p = 0.008	R = -0.469 p = 0.037
Serum FSH (MIU/ml)	r = 0.05 p = 0.84	R = - 0.268 p = 0.25

Statistically significant at $p < 0.05$

In addition, Gualillo et al. (2001) demonstrated that ghrelin m-RNA and ghrelin peptides are present in human and rat placenta indicating that ghrelin may have some role not only in maternal circulation but also in fetal-placental circulation. Moreover, Cortelazzi et al. (2003) found the association between high ghrelin levels and intrauterine growth retardation fetuses.

An earlier study demonstrated a positive association between ghrelin levels with current smoking in a population of 58 years old healthy men (*Fagerberg* et al. 2003), while Bouros et al. (2006) found increased ghrelin levels as a response to acute effects of smoking. They concluded that the effects of smoking on appetite could be mediated by ghrelin. Contrary to our findings, Kokkinos et al. (2007) observed that total ghrelin levels were not influenced by long term smoking as baseline plasma ghrelin were not changed between smokers and non smokers.

Smoking associated hyperghrelinemia could be related to its adverse effects on gastric mucosa, gastric motility, mucosal blood flow and concentrations of free radicals in the cigarette smoke (Cummings et al. 2002). The enhancement of ghrelin could be also an indirect influence mediated by other factors including growth hormones and leptin. Reduced leptin might represent a secondary effect of smoking induced by increased catecholamines which are known to reduce leptin expression in vitro and in vivo. Adrenaline has been found to increase the transport of leptin over the blood brain barrier by increasing the uptake and brain/serum ratio of leptin (Reseland et al. 2005).

Among the wide range of ghrelin actions, fragmentary evidences suggest that ghrelin might regulate the network controlling gonadal function and the gonadotropic axis, thereby contributing to the physiological systems linking energy status and reproduction. (Martini et al. 2006; Zouc et al. 2008).

Garcia et al. (2007) initiated the analysis of ghrelin pattern and its cognate receptor in rat testis, by a combination of molecular and immunological approaches. Testicular ghrelin expression was demonstrated by PCR throughout postnatal development with the highest ghrelin levels observed in rat Leydig cells during the adult period 60–90 days old (Tena-Sempere et al. 2002).

In the present study, the adult male offsprings exposed to CS *in utero* demonstrated hyperghrelinemia in addition to reduced testicular weight and low serum and testicular testosterone in comparison to non exposed offsprings. This hyperghrelinemia could be suggested to inhibit testicular hormones, which is clearly evident from the negative correlation obtained between ghrelin levels and both serum and testicular testosterone.

The biological actions of ghrelin in testis are carried out through the interaction with its receptor GHS-R1a. Thus, in Leydig cells, the overexpression of ghrelin thus interacting with GHS-R1a may lead to the inhibition of testosterone (Ishikawa et al. 2007). It was previously mentioned that the inhibitory effect of ghrelin *in vitro* upon testosterone secretion was associated with a significant decrease in human chorionic gonadotropin (hCG)- stimulated levels of the mRNAs encoding several key factors in the steroidogenic route, such as steroid acute regulatory protein (StAR), and the enzymes P450, 3 beta- hydroxysteroid dehydrogenase (HSD) and testis-specific 17 -beta HSD (Barreiro and Tena-Sempere 2004).

Some evidences indicated the ghrelin might also directly regulate seminiferous tubules functions, as expression of GHS-R1a was demonstrated in the tubular compartment of the testis (Kheradmand et al. 2009).

Barreiro et al. (2004) demonstrated that intratesticular injection of ghrelin (15 µg for 2 days in adult rats) inhibited the expression of gene encoding stem cell factor (SCF), a key signal in spermatogenesis and putative regulator of Leydig cell development. Such an inhibitory action was also detected *in vitro* using cultures of staged seminiferous tubules. Notably SCF is a Sertoli cells product that has been identified as the major paracrine stimulator of germ cell development acting as a survival factor for spermatogonia, spermatocytes and spermatids in adult rat seminiferous epithelium (Garcia at al. 2007). The gonadotropin FSH was reported to be the major regulator of SCF since receptors of FSH are solely expressed in Sertoli cells (Hakovitra et al. 1999).

In this work, the exposed rats demonstrated suppression of pituitary gonadotropines (LH and FSH) as compared to non exposed controls. In addition, a negative association was noticed between high ghrelin and both LH and FSH in the same group indicating an extra gonadal action of ghrelin upon reproductive axis.

In rats, limited number of studies were carried out to analyze the effects of ghrelin on the pituitary secretion of hormones other than GH (Fernandez et al. 2004). The regulation of gonadotropin secretion is carried out through a complex interaction between hypothalamic luteinzing hormone releasing hormone (LHRH), other hypothalamic peptides, locally produced pituitary signals such as activin, inhibin, gonadal derived peptides and steroids (Moore et al. 2003).

The presence of ghrelin receptors in the hypothalamus and pituitary gland opens the possibility that ghrelin participates in the control of pituitary secretion and in addition to its effects on GH, ghrelin actions on prolactin and LH release have been reported. It was demonstrated that intracerebroventricular injection with ghrelin (3 nmol/rat) in pubertal intact and gondadectomized rats resulted in inhibition of LH secretion in vivo where as FSH remained unaffected. (Fernandez et al. 2004). More recently, Fernandez et al. (2005), found that daily subcutaneous administration of ghrelin to prepubertal male rats induced a significant decrease in serum testosterone during the study period, which was associated with normal to decreased LH levels. The normal occurrence of preputial separation (external index of puberty) was prevented in more than 40 % of ghrelin treated rats. They explained that the mechanism for such an effect might involve the inhibition of LH secretion at the hypothalamic-pituitary unit or direct inhibitory effects on the testicular testosterone secretion. The fact that testosterone levels were lower in ghrelin treated animals strongly suggests a contribution of ghrelin on the prepubertal testis. These might include disturbance of adult type Leydig cells in pubertal testis because ghrelin has been recently proven to inhibit the proliferative activity of the immature Leydig cells in pubertal testis. Moreover, the study of Martini et al. (2006) showed that the repeated infusion of low ghrelin doses for ten days throughout the puberty decreased LH levels, while the same in adult male rats resulted in significant decreases in circulating LH and FSH concentrations.

Since the secretion of FSH and testosterone is necessary for spermatogenesis cycle, therefore in our study, restricted sperm production after smoking induced hyperghrelinemia is expectable. This is clearly evident from the negative correlation between ghrelin and FSH.

It is well known that the programmed developmental transitions (proliferation/apoptosis and differentiation) of the Leydig cells are critical for testosterone production. In addition to a pituitary trophic regulation, existence of multiple self regulatory signaling systems is important for finely tuning the functions within the testis (Kumar 2004).

In conclusion, data of the present study support the concept that ghrelin participates in the control of pituitary hormones other than GH and suggest that the elevated ghrelin as a result of smoking might be detrimental for normal puberty and testicular function. Considering that circulating ghrelin levels are negatively correlated with body weight and that the proposed role of ghrelin as a signal for energy insufficiency, it is tempting to propose that ghrelin *per se* might operate as a negative modifier of pituitary gonadal axis and it might contribute to the suppression of reproductive function in situations of negative energy balance such as in smoking. Moreover, the findings of this study provide the evidence that maternal smoking during pregnancy could affect the hormones influencing appetite such as ghrelin. Changes in ghrelin levels and its effect on pituitary gonadal axis after quitting the smoking are needed to be investigated. Further studies are also warranted to clarify the effect of smoking on various gut hormones.

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