

Relationship between ghrelin and estrogen in the ovary of pregnant sheep

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Abstract:

BACKGROUND: Ghrelin, an endogenous ligand for the growth secretagogue receptor is predominantly produced in the stomach and an expression of ghrelin has recently been identified in placenta and ovary. **OBJECTIVES:** Therefore, we decided to measure ovarian ghrelin as quantitative and evaluate the correlation between ghrelin and estrogen during second half of ovine pregnancy. **METHODS:** The ovarian samples were collected from 40 pregnant sheep in 3, 3.5, 4, 4.5 and 5 months of pregnancy in a local abattoir. Follicles and active corpora lutea (CL) were dissected from surrounding tissues, separately. The samples were homogenized with phosphate buffer (0.1 M, pH=7.4) on liquid nitrogen to provide fluid samples. Ghrelin and estrogen concentrations were measured by ELISA method and expressed as milligram (mg) and picogram (pg) per mg of tissue protein, respectively. **RESULTS:** There was a linear correlation between ghrelin and estrogen in ovarian follicles ($r=0.97$ and $p=0.004$), but not in CL. Follicular ghrelin significantly increased in 4, 4.5 and 5 months ($p<0.001$) but CL ghrelin significantly decreased in the 4.5 month of ovine pregnancy ($p<0.01$). Estrogen concentration was also significantly higher in 4-5 months of pregnancy in ovarian follicles ($p<0.001$) but potent CL indicated higher estrogen level only in the 5th month of pregnancy ($p<0.001$). **CONCLUSIONS:** These results emphasize the role of ghrelin in the reproductive system and open a new window to future studies.

Introduction

In recent years, there has been increasingly more evidence to support the biological actions of ghrelin than those originally anticipated (Alirezaei et al., 2015; Du et al., 2010; Dupont et al., 2010; Du et al., 2009; Rak and Gregoraszczyk 2008; Caminos et al., 2003). Ghrelin has been identified as an endogenous ligand for growth hormone secretagogue receptor (GHSR) that regulates growth hormone

(GH) secretion, regulates food intake, increases appetite and contributes to insulin release and energy homeostasis as well as acting as an antioxidant peptide (Alirezaei et al., 2015; Kheradmand et al. 2010, 2011; Neamati et al. 2011; Kojima and Kangawa 2005; Obay et al. 2008). Expression of ghrelin has been identified in an array of tissues and cell types including the stomach, small intestine, pancreas, lymphocytes, placenta, kidney, lung, pituitary and brain (Gualillo et al., 2003, Du et al., 2009).

This ubiquitous distribution suggests that locally produced ghrelin might exert paracrine/autocrine effects in different tissues (Du et al., 2010). So, this may be the case in various reproductive organs such as endometrium, placenta, testis and ovary (Du et al., 2010; Garcia et al., 2007; Harrison et al., 2007). Recently, new evidence strongly indicated that the ghrelin and ghrelin receptors (GHS-R1a, GHS-R1b) are present in the mammalian and non-mammalian ovary (Dupont et al., 2010) and expression of ghrelin mRNA in ovarian tissue has been reported (Rak and Gregoraszczuk 2008; Caminos et al., 2003; Gnanapavan et al., 2002). In this regard, ghrelin is found in human, rat, pig and sheep ovary (Dupont et al., 2010; Zhang et al., 2008; Sirotkin et al., 2006; Miller et al., 2005; Caminos et al., 2003; Gaytan et al., 2003). Ghrelin is expressed during the estrous cycle and pregnancy and the relative mRNA levels depend on the stage of the cycle with the highest expression during the development the corpora lutea in sheep ovary (Dupont et al., 2010). Likewise, strong ghrelin immunostaining was observed in ovarian follicles at all development stages (Miller et al., 2005; Du et al., 2009). These results encouraged the researchers to provide further evidence regarding the role of this hormone in reproduction of domestic animals.

It is well known that the ovary is a complex organ in which different endocrine and locally produced steroidal and non-steroidal regulators cooperate to ensure complete ovarian function (Rak and Gregoraszczuk 2008). It has been recently shown that a factor that directly regulates ghrelin gene expression in the stomach of rats is estrogen, which plays a critical role in ghrelin expression and production (Sakata et al., 2006). This finding confirmed that the produced estrogen in the stomach regulates ghrelin gene expression and the published data have demonstrated that ghrelin is produced by the ovarian follicle, increases estradiol secretion and influences aromatase activity in pre-

pubertal pig ovaries (Rak and Gregoraszczuk 2008). Our previous report also demonstrated changes of aromatase activity (a key enzyme for estrogen synthesis) in follicles and corpora lutea during the second half of ovine pregnancy (Sookhtehzari et al., 2011). In order to better understand the potential relationship between ghrelin and estrogen, the concentrations were measured in follicles and corpora lutea per milligram of tissue protein. Hence, the aim of the present study was to evaluate the relationship between ghrelin and estrogen during the second half of ovine pregnancy.

Materials and Methods

Animals and tissue collection: A total of 120 pregnant sheep (24-48 months of age) were used in this study from November 2014 to January 2015 at the abattoir in Lorestan province, Khorram Abad, Iran. The stage (month) of pregnancy was estimated by measuring foetal size, based on the crown-rump length of the fetus (Sivachelvan et al., 1996). The ovarian samples were collected from the pregnant sheep using a pair of surgical scissors for 3, 3.5, 4, 4.5 and 5 months of pregnancy, immediately after slaughter at the local abattoir. The ovaries were transported at ice-cold temperature to the biochemical laboratory. All follicles were collected from the surface with a surgical blade and functional corpora lutea (CL) was dissected from the 40 ovarian samples in order to provide 8 samples for each stage of pregnancy (5 stage \times 8 samples=40). The samples were stored at -70 °C for a maximum of up to 2 months prior to biochemical analysis.

Tissue preparation and biochemical analysis: The tissues were thawed and manually homogenized in cold phosphate buffer (0.1 M, pH=7.4) on liquid nitrogen as described previously (Alirezai et al. 2015) and debris were removed by centrifugation at 2000 RPM for 20 min (Centrifuge 5415 R; Rotofix 32A, Germany). Supernatants were recovered and used

for protein measurement, ghrelin content and estrogen concentration. Protein content of tissue homogenates was determined using a colorimetric method of Lowry with bovine serum albumin as a standard (Lowry et al., 1951).

Ghrelin measurement: Ghrelin content was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Bioassay Technology Laboratory, Shanghai, China) based on the biotin double antibody sandwich technology via an ELISA reader (STAT FAX, 2100, USA) at 450 nm. This kit was prepared to assay the ghrelin in the sample of sheep's serum, blood samples and other related biological fluids. The detectable level of this kit was 20 to 320 ng/l and the coefficients of inter-and intra-assay precision were <12 % and <10 %, respectively. The ghrelin concentration was expressed as mg/mg protein of tissue homogenates (mg/mg of tissue protein).

Estrogen measurement: Estrogen concentration was assayed by the competitive ELISA method according to the manufacturer's instructions kit (Dia Metra, Foligno (PN), Italy), as described previously in our laboratory (Sookhtehzari et al., 2011). In brief, 25 µl of the each E2 standard (0, to 2000 pg/ml in duplicate, for standard curve preparation) and 25 µl of tissue homogenates were transferred to the anti-E2 IgG-coated wells of the plate. The immunoreaction was initiated by adding 200 µl of the E2-HRP conjugate solution to each well, followed by incubation at 37 °C for 2 h. The content from each well was removed and washed with 300 µl of distilled water, 5-6 times. The water was completely drained out from each well. Next, 100 µl of TMB substrate was dispensed into each well and incubated at 25 °C for 30 min in dark condition. Color development was stopped with 100 µl of stop solution. Absorbance was taken at 450 nm using an ELISA reader. Calculation from the measured absorbance of the samples was interpolated from the standard curve to obtain the values. The cross reactivity of the antibody

used, calculated according to manufacturer's instructions kit were: estradiol 100%, estrone 2%, estriol 0.39%, and testosterone 0.02%. Estrogen concentration was expressed as pg/mg of tissue homogenates (pg/mg of tissue protein).

Statistical analysis: Statistical analysis was performed using the statistical package GraphPad PRISM version 5 (GraphPad Software Inc., San Diego, CA, USA). All variables were tested for normal and homogeneous variances by Leven's statistic test. All results are presented as mean± (S.E.M.). The statistical differences were applied among all stages of pregnancy by one-way analysis of variance (ANOVA) with Tukey's post hoc analysis. Pearson's correlation and linear regression tests were also used between ghrelin and estrogen concentrations in follicles and corpora lutea. A calculated P value of less than 0.01 was considered statistically significant.

Results

Values represent Mean ± SEM of estrogen and ghrelin concentrations in follicles and corpora lutea during second half of ovine pregnancy. Figure 1 indicates that estrogen concentration in follicles significantly increased in the months 4, 4.5 and 5 in comparison with 3 and 3.5 months of ovine pregnancy ($p<0.001$) and there was a tendency to increase follicular estrogen from 4 to 5 months in pregnant sheep. There was also a significant increase of follicular ghrelin in 4, 4.5 and 5 months of pregnancy when compared to both 3 and 3.5 months in pregnant sheep ($p<0.001$, Figure 2). The estrogen concentration of active CL significantly increased in 5th month when compared to other months ($p<0.001$; Figure 3). In contrast, there was a significant decrease in ghrelin concentration for the 4.5 month of pregnancy as compared to the 5th month in functional CL ($p<0.0$; Figure 4).

Regarding correlation between ghrelin and

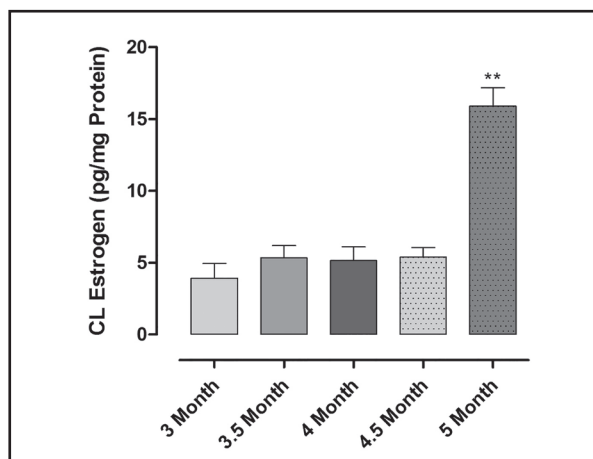


Figure 1. Estrogen concentration in follicles during second half of ovine pregnancy. Values represent mean \pm SEM of estrogen (pg/mg protein of follicle). **Asterisk indicates statistical difference versus 4, 4.5 and 5 months of pregnancy ($p < 0.001$).

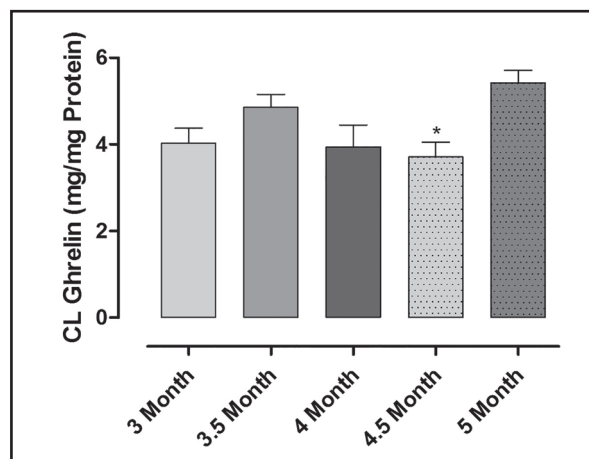


Figure 2. Ghrelin concentration in follicles during second half of ovine pregnancy. Values represent mean \pm SEM of ghrelin (mg/mg protein of follicle). **Asterisk indicates statistical difference versus 4, 4.5 and 5 months of pregnancy ($p < 0.001$).

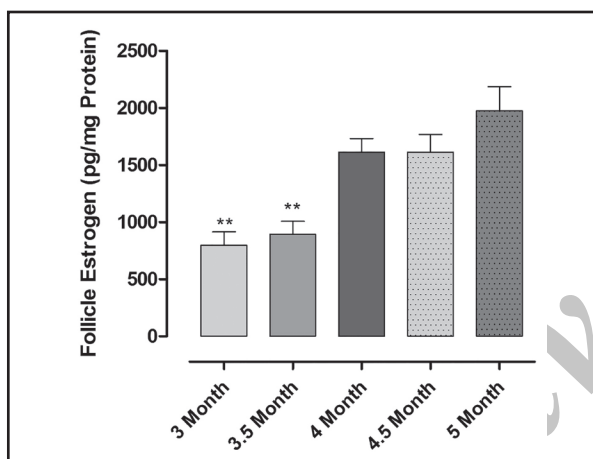


Figure 3. Estrogen concentration in corpus luteum (CL) during second half of ovine pregnancy. Values represent mean \pm SEM of estrogen (pg/mg protein of CL). **Asterisk indicates statistical difference versus 3, 3.5, 4 and 4.5 months of pregnancy ($p < 0.001$).

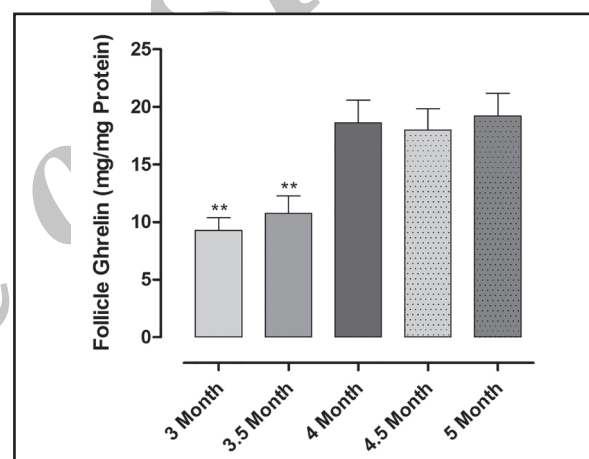


Figure 4. Ghrelin concentration in corpus luteum (CL) during second half of ovine pregnancy. Values represent mean \pm SEM of ghrelin (mg/mg protein of CL). *Asterisk indicates statistical difference versus 5 month of pregnancy ($p < 0.01$).

estrogen concentrations in follicles and corpora lutea, there was a positive linear regression between follicular estrogen and ghrelin during second half of ovine pregnancy ($r=0.97$ and $p=0.004$), while there was no correlation between estrogen and ghrelin in corpus luteum ($p>0.05$).

Discussion

This report is probably the first to show the ghrelin and estrogen relationships during

the second half of ovine pregnancy. Our data demonstrate that follicular estrogen concentration increases during second half of ovine pregnancy concomitant with ghrelin concentration. This effect is more noticeable for 4, 4.5 and 5 months in pregnant sheep. Based on the present results, the estrogen concentration in corpus luteum (CL) was only significantly higher in the 5th month of pregnancy as compared to other months. In contrast, the ghrelin concentration in CL significantly decreased in the 4.5 month when compared to the 5th

month in pregnant sheep. Therefore, it seems there is a relation between follicular estrogen and ghrelin during second half of pregnancy in the sheep ovary.

As previously mentioned, recent evidence strongly indicates that the ghrelin signal is present in the ovary (Du et al., 2009; Zhang et al., 2008; Miller et al., 2005; Caminos et al., 2003; Gaytan et al., 2003). In the present study, ghrelin concentration within sheep ovarian tissue revealed distribution of ghrelin in both follicles and CL. One of the interesting findings of the current study was the positive correlation between ghrelin and estrogen in ovarian follicles. Based on the results, ghrelin levels are increased when estrogen concentrations are strongly increased from the 4th to 5th month of pregnancy. In this setting, it has been found that estrogen treatment significantly simulated ghrelin mRNA expression and ghrelin production in a dose-dependent manner, indicating that estrogen works on ghrelin gene expression through the α -estrogen receptor (Sakata et al., 2006). Our data showed a significant relation between ghrelin and estrogen in the ovarian follicles. To our knowledge, the source of supply of estrogen precursors is considered to be the gonads or adrenal gland (Lu and Judd 1982; Sakata et al., 2006). Recent studies evidenced that androgens were independent modulators of ghrelin level in the gonads, thus confirming an interaction between ghrelin and sex estrogen synthesis (Rak and Gregoraszczuk 2008; Gambiner et al., 2003; Pagotto et al., 2002). It is well known that aromatase activity, as key enzyme, plays a critical role in estrogen synthesis (Caminos et al., 2003). Moreover, there was a close relation between ghrelin cells and aromatase-expressing cells in the follicular wall, suggesting that ghrelin cells are exposed to estrogen (Du et al., 2010). Therefore, the estrogen concentration increased concomitant with ghrelin level in follicles. In this regard, a previous work demonstrated dependent action of ghrelin on estradiol secretion by fol-

licular cells (Gregoraszczuk et al., 2000). The above and the previous report (Rak and Gregoraszczuk 2008) confirm that ghrelin stimulates estradiol secretion by ovarian follicles and acts on aromatase activity. Therefore, we described a notably dynamic pattern of follicular ghrelin and estrogen with the lowest concentration in 3 and 3.5 months and maximum values in 4, 4.5 and 5 months in pregnant sheep.

To evaluate the relation between ghrelin and estrogen in the CL, measurement of ghrelin and estrogen levels was conducted throughout the 3-5 months of pregnancy in an active CL. Interestingly, despite a persistent decrease of the estrogen concentration in 3-4.5 months of pregnancy, estrogen level significantly increased in the 5th month of pregnancy in potent CL. Indeed, estrogen level reached its highest value when CL entered into its regression phase. Our previous report indicated a decrease of aromatase activity in CL during second half of ovine pregnancy and there was a negative correlation between aromatase activity and stage of pregnancy ($r=-0.69$ and $p<0.05$) (Sokhtehzari et al., 2011). Al-Gubory et al., 1994 also reported that a non-steroidal factor in the ovine CL of late pregnancy directly inhibits aromatase activity in ovarian follicles. Therefore, it seems that the increasing of estrogen concentration is related to the blood estrogen level that was originated from the placenta or elsewhere. In this regard, there was no correlation between ghrelin and estrogen in CL which indicates ghrelin elevation in the 5th month also is related to elevation of blood estrogen since estrogen directly induces ghrelin gene expression and production (Sakata et al., 2006). The previous reports on the biochemistry and structure of the CL, suggest that during late pregnancy the CL is at the very early stage of structural regression, with no changes at the morphological level, but with changes in the molecular and biochemical functions (Du et al., 2009; Caminos et al., 2003). Hence, ghrelin levels decreased in CL at 4.5 month of

pregnancy, along with the regression of CL. In the Figure 4, we demonstrated slightly higher ghrelin content for 3.5 months in comparison with 4, and 4.5 months in pregnant sheep, although it was not significant. This result is in agreement with Du et al's., 2009 study in which middle CL (mature CL) indicated higher relative mRNA expression of ghrelin when compared to late CL (Du et al., 2009). It seems elevations of ghrelin and estrogen in late pregnancy are partially related to blood estrogen when estrogen concentration increases at the late stage of pregnancy to induce parturition. Nevertheless, it remains to be established whether ghrelin is actually involved in estrogen synthesis of the CL during 3-5 months of pregnancy.

In conclusion, ghrelin peptide is indicated as quantitative in ovarian follicles and functional CL during pregnancy of sheep. Dynamic changes in the profile of ovarian ghrelin and estrogen were more noticeable during second half of ovine pregnancy. These results further emphasize the potential regulatory role of ghrelin in the reproductive system and open a new window to future studies.

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رابطه بین گرلین و استروژن در تخمدان گوسفندان آبستن

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چکیده

زمینه مطالعه: گرلین، بعنوان یک لیگاند داخلی برای گیرنده ترشحی رشد بطور غالب در معده تولید می گردد و اخیراً بیان گرلین در جفت و تخمدان گزارش شده است. **هدف:** بنابراین ما تصمیم گرفتیم که گرلین تخمدانی را بصورت کمی ارزیابی و رابطه گرلین و استروژن را در طول نیمه دوم آبستنی در گوسفند بررسی کنیم. **روش کار:** نمونه های تخمدان از ۴۰ میش آبستن در ۳، ۴، ۵، ۴/۵ و ۵ ماهگی از آبستنی در یک کشتارگاه محلی جمع آوری شدند. فولیکول ها و جسم زرد فعال از بافت های اطراف بصورت جداگانه تهیه گردیدند. نمونه ها با استفاده از بافر فسفات (یک دهم مولار با $pH=7.4$) بر روی ازت مایع هموژنیزه شدند تا نمونه های مایع بدست آید. غلظت گرلین و استروژن بوسیله روش الیزا اندازه گیری شد و بترتیب بصورت میلی گرم و پیکوگرم در میلی گرم از پروتئین بافت برای گرلین و استروژن بیان گردید. **نتایج:** یک رابطه خطی بین گرلین و استروژن در فولیکول های تخمدان وجود داشت ($p=0.004, r=0.97$) اما در جسم زرد این رابطه نبود. گرلین فولیکول ها بطور معنی داری در ۴، ۵ و ۴/۵ ماهگی افزایش یافت ($p<0.001$) ولی گرلین جسم زرد بطور معنی داری در ۴/۵ ماهگی از آبستنی گوسفند کاهش یافت ($p<0.001$). در فولیکول های تخمدان همچنین غلظت استروژن در ۵-۴ ماهگی آبستنی بالا بود ($p<0.001$) اما جسم زرد فعال فقط در ۵ ماهگی غلظت استروژن بالاتری نشان داد ($p<0.001$). **نتیجه گیری نهایی:** این نتایج نقش گرلین را در سیستم تولید مثل برجسته می کند و دریچه جدیدی رو به مطالعات آینده باز می کند.

واژه های کلیدی: جسم زرد، استروژن، فولیکول، گرلین، گوسفند

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