

## The effect of ghrelin on Kiss-1 and KissR gene transcription and insulin secretion in rat islets of Langerhans and CRI-D2 cell line

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### ABSTRACT

**Objective(s):** Ghrelin is a peptide hormone that has been shown to have numerous central and peripheral effects. The central effects including GH secretion, food intake, and energy homeostasis are partly mediated by Kiss1- KissR signaling pathway. Ghrelin and its receptor are also expressed in the pancreatic islets. Ghrelin is one of the key metabolic factors controlling insulin secretion from the islets of Langerhans. We hypothesize that the inhibitory effect of ghrelin on KiSS-1 and KissR in the islet cells may be similar to the same inhibitory effect of ghrelin in the hypothalamus.

**Materials and Methods:** To investigate the effect of ghrelin, we isolated the islets from adult male rats by collagenase and cultured CRI-D2 cell lines. Then, we incubated them with different concentrations of ghrelin for 24 hr. After RNA extraction and cDNA synthesis from both islets and CRI-D2 cells, the relative expression of KiSS-1 and KissR was evaluated by means of real-time PCR. Furthermore, we measured the amount of insulin secreted by the islets after incubation in different concentrations of ghrelin and glucose after 1 hr. Besides, we checked the viability of the cells after 24 hr cultivation.

**Results:** Ghrelin significantly decreased the KiSS-1 and KissR mRNA transcription in rat islets and CRI-D2 cells. Besides, Ghrelin suppressed insulin secretion from pancreatic beta cells and CRI-D2 cells.

**Conclusion:** These findings indicate the possibility that KiSS-1 and KissR mRNA expression is mediator of ghrelin function in the islets of Langerhans.

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### Introduction

Ghrelin is a 28 amino acid peptide is secreted mainly from the stomach. It is an endogenous ligand of the growth hormone receptor (1) that was later renamed ghrelin receptor (GRLN-R) (2). This hormone has various biological functions, prominently on the regulation of food intake, gastrointestinal motility, and energy homeostasis (3). The orexigenic effect of ghrelin is achieved by passing through the blood-brain barrier and its action on food regulatory brain nuclei (4). Ghrelin is also produced centrally in the arcuate nucleus of the hypothalamus which has a key role in regulation of food intake (5). Recently it has been established that ghrelin suppresses the pulsatile luteinizing hormone (LH) secretion from the hypothalamic medial preoptic area by suppression of Kiss-1 gene (6).

The expression of KiSS-1 in the hypothalamus is sensitive to nutritional state, and it might contribute to the suppression of reproductive function in such conditions as negative energy balance periods (7).

The Kiss-1 gene encodes 54, 14, 13, or 10 amino-acid peptides, well known as kisspeptins (8) Kisspeptins has been detected in the central nervous system as well as peripheral tissues such as placenta, testes, and pancreas (9). Kisspeptin (Kiss1) and its G protein-coupled receptor (GPR-54) (Kiss1r) is an essential component of the controlling ghrelin expression in the hypothalamus. Since pancreatic beta cells also express KiSS-1 and kissR, it raises the possibility that ghrelin may have a similar role in the transcription of kiss1-KissR signaling in the pancreas too. Ghrelin and its receptor have been detected in other peripheral tissues, including pancreas (10).

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Actually, ghrelin and its receptor mRNAs and proteins have been identified in both human and rat pancreatic islets (11).

The exact role of ghrelin in regulation of insulin secretion is not definitely understood. Ghrelin was found to inhibit insulin secretion in some experiments but to stimulate it in others. Ghrelin inhibited glucose-stimulated insulin secretion from isolated human and rat islets (12) and immortalized  $\beta$ -cell lines (13). In contrast, ghrelin increased the cytosolic free  $Ca^{2+}$  concentration and stimulated insulin secretion when it was added to isolated rat islets (14). So we evaluate the effect of different concentrations of ghrelin on insulin secretion in rat islets of Langerhans and an insulinoma cell line (CRI-D2) as a pure beta cell source.

## Materials and Methods

### Islets isolation

In this study, adult Wistar male rats weighing 300–350 g were maintained under 12 hr light, 12 hr dark conditions at 22–24 °C and had free access to pelleted food and tap water.

All the animal experiments were approved by the Departmental Committee for Care and Use of Laboratory Animals, Shiraz University of Medical Sciences, Shiraz, Iran.

Anesthesia was provided using Ketamin (100 mg/kg IP) and rat islets of Langerhans were isolated by collagenase P (Roche, Germany). Besides, pancreatic duct was cannulated with PE50 tube (Becton Dickinson Company) and collagenase P (15 mg in 15 ml HBSS (Hank's Balance Salt Solution)) was injected into the duct. The distended pancreas was maintained at 37 °C for 25 min. Then, the islets were washed by medium A (HBSS, 1% HEPES (Sigma-Aldrich, USA), 2%FBS) 5 times and by means of Lymphoprep (Axis-Shield, Norway); the islets were isolated and purified from exocrine cells. Islet size and purity were determined by microscopic sizing on a grid after staining with 1, 5-diphenylthiocarbazon (DTZ). Afterward, the islets were incubated in 5%  $CO_2$  in RPMI 1640 (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotic (Invitrogen) at 37°C for 24 hr before use.

### Cell culture

To investigate whether the transcription of KiSS-1 and KissR genes could be directly regulated by ghrelin, we used rat islets of Langerhans and CRI-D2 cell line. We used an insulinoma cell line (CRI-D2) as a pure beta cell source while islets are a complex of different cells which secrete different hormones. CRI-D2 cell line was supplied by national cell bank of Iran (NCBI), Pasteur Institute (Tehran, Iran) and the detailed profile is provided at <http://hpcultures.org.uk>. The islets were seeded into 48-well plates at 100 islets per well and the CRI-D2 cell line was placed in 6-well plates at  $10^5$  cells per well and incubated at 37 °C in an atmosphere of 5%

$CO_2$  in RPMI supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin for 24 hr before use.

### Islets treatment with ghrelin

After 24 hr incubation, the medium was replaced with medium alone or containing  $10^{-6}$ ,  $10^{-8}$ ,  $10^{-9}$ ,  $10^{-11}$  M ghrelin (Sigma-Aldrich, USA) in each well and then incubated for 24 hr. These concentrations were selected on the basis of previous references (20).

### RNA isolation and cDNA synthesis

Total RNA was extracted from the islets and the cell line by RNA kit II (Invitex, Germany) according to the manufacturer's instructions. The extracted RNA was quantitated by  $OD_{260/280}$  measurement. Moreover, the total RNA (10  $\mu$ g) of the cell culture extracts was reverse transcribed in a 20- $\mu$ l volume using random hexamer primers, with enzyme and buffers supplied by the cDNA First Strand Synthesis kit (Fermentas, Life Science, EU).

### Quantitative real-time PCR

At first, 5  $\mu$ g of cDNA was added to taq man master mix (TaKaRa, Takara Shuzo., Otsu, Japan). The final volume of the PCR was 20  $\mu$ l: 10  $\mu$ l Master Mix, 0.6  $\mu$ l of each primer, 0.6  $\mu$ l probe, 0.4  $\mu$ l reference dye, and 2.8  $\mu$ l  $dH_2O$ . Amplification of DNA was performed under the following conditions: 10 min at 95 °C, 10 sec at 95 °C, and 30 sec at 60 °C for 40 cycles. The primers and probes for real-time PCR were designed using rat genomic sequences as templates through NCBI (<http://www.ncbi.nlm.nih.gov/pubmed>) and Allele ID programs. Furthermore, GAPDH was selected as the endogenous control and the transcription of Kiss-1 and Kiss receptor was checked relative to GAPDH.

The mRNA expression was quantified using the comparative cross threshold (CT) method. The CT value of the housekeeping gene (GAPDH) was subtracted from the CT value of the target genes to obtain  $\Delta$ CT. The normalized fold changes of mRNA expression were expressed as  $2^{-\Delta\Delta CT}$  where  $\Delta\Delta CT$  equals to  $\Delta CT$  sample –  $\Delta CT$  control.

Each experiment was repeated 3 times.

The sequences of the primers and probes are listed in Table 1.

**Table 1.** Sequences of the primers and probes

	Forward primer	Reverse primer	Probe
GAPDH	GGCTCTCTGCT CCTCCCTGTTC	CGGCCAAATCCGTT CACACCGA	GCCGCATCTTCTT GTGCAGTGCCAGCC
KiSS-1	ATGATCTCGCT GGCTTCTTGG	GGTTCAGGGTT CACCACAGG	TGCTGCTTCTCTCT GTGTGGCCT
kissR	TTCCTCTCTGT GCTGCGTACC	CGAGACCTGCTGGAT GTAGTTG	CGCTCTCTATCCGCT GCCACCT

### Insulin secretion in vitro

The effect of ghrelin on insulin secretion was assessed by static incubation of the islets. Groups of 20 islets (hand-picked) were placed in 6-well plates and incubated in Krebs-Ringer-HEPES (KRH) buffer (125 mM NaCl, 4.74 mM KCl, 1 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 5 mM NaHCO<sub>3</sub>, 25 mM HEPES, 0.1% BSA, pH 7.4) containing 2.8 mM glucose (Sigma-Aldrich, USA). After 1 hr of pre-incubation, the supernatant was collected and the cells were stimulated with KRH buffer containing 8.3 mM glucose for 1 hr. Again, the supernatant was collected.

This procedure was repeated with KRH buffer containing 16.7 mM glucose. To test the effects of ghrelin, this peptide was applied during the 1 hr incubation period at the given glucose concentration. We used 10<sup>-6</sup>, 10<sup>-11</sup> M ghrelin according to the previous studies in rat islets. Insulin in the supernatant was determined by rat insulin ELISA kit (Merckodia, Sweden).

### Cell apoptosis

In order to examine whether the islets and cells underwent apoptosis during the incubation period, we checked them for apoptosis.

Groups of 20 islets or 10<sup>4</sup> CRI-D2 cells were treated with ghrelin 10<sup>-7</sup>, 10<sup>-11</sup> M and without ghrelin or (control) for 24 hr. After incubation at 37°C, apoptosis of the samples was investigated using the In Situ cell death detection kit POD (Roche, Germany) and interpreted according to the protocol.

### Statistical analysis

The data are shown as mean ± SD. For evaluation of each gene transcription, 3–5 test groups were compared to the controls and each experiment was repeated 3 times.

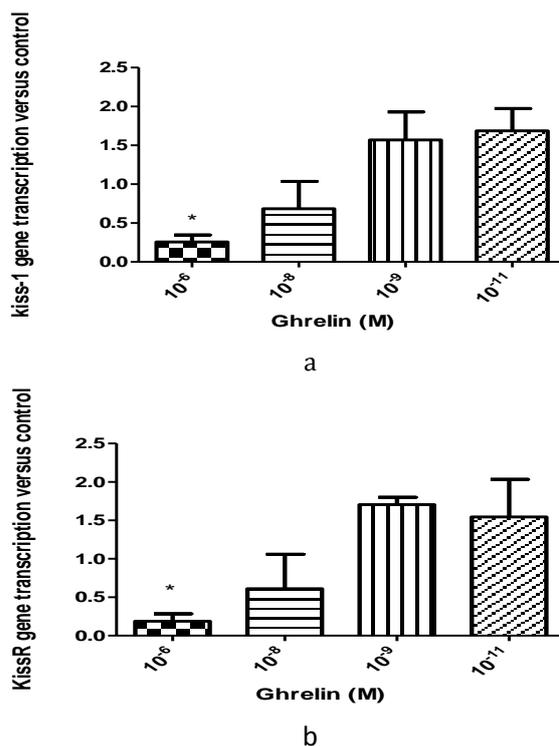
Different groups were compared using one-way ANOVA followed by Tukey's test for pairwise comparison.

For the insulin secretion, groups were compared using two-way ANOVA. A *P*-value < 0.05 was considered as statistically significant. The statistical analysis and design of the graphs were performed using Graph Pad Prism 5 software.

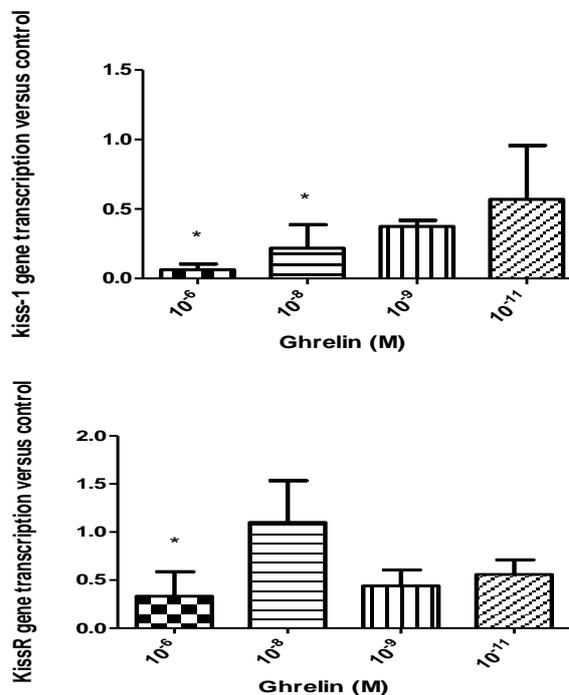
## Results

### The effect of ghrelin on Kiss-1 and KissR mRNA transcription in rat islets of Langerhans

In order to determine the effect of ghrelin, first, we investigated its effect on rat islets of Langerhans. Ghrelin (10<sup>-6</sup>M) significantly decreased the transcription of Kiss-1 compared to the control group (*P*-value < 0.05) in the islets of Langerhans, this concentration of ghrelin significantly diminished KissR transcription in islet cells too (Figure 1 a,b). The effects of other concentrations of ghrelin were not significant.



**Figure 1, a)** The effects of different concentrations of ghrelin on Kiss-1 transcription in rat islets of Langerhans, **b)** The effect of ghrelin on KissR transcription in rat islets of Langerhans. All data are shown as mean ± SD, Analysis by one-way ANOVA, \**P*-value < 0.05



**Figure 2, a)** The effect of different concentrations of ghrelin on Kiss-1 transcription in CRI-D2 cells, **b)** The effect of ghrelin on KissR transcription in CRI-D2 cells. All data are shown as mean ± SD, Analysis by one-way ANOVA, \**P*-value < 0.05

**The effect of ghrelin on KiSS-1 and KissR mRNA transcription in CRI-D2 cells**

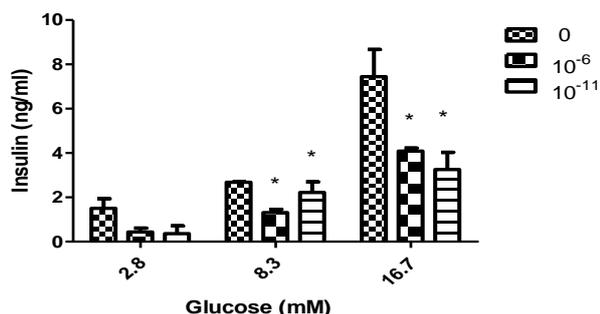
The higher level of ghrelin (10<sup>-6</sup>M) was also most effective in CRI-D2 cells. The same inhibitory results acquired in transcription of Kiss-1 from the CRI-D2 cells and it was significant for 10<sup>-6</sup>M ghrelin (P-value<0.05). In the transcription of KiSS-1, both 10<sup>-6</sup>M and 10<sup>-8</sup>M were significant (P-value<0.05) (Figure 2 a,b)

**The effect of ghrelin on insulin secretion in the islets of Langerhans**

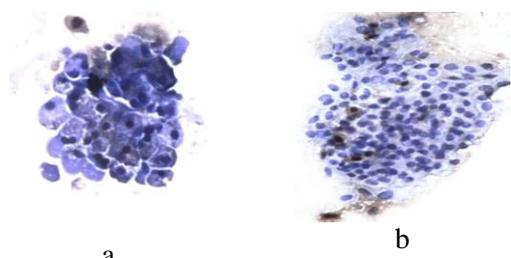
Next, we applied two effective ghrelin concentrations to the isolated islets culture plate, and insulin was subsequently measured. Insulin secretion was significantly increased with 8.3 and 17.6 mM glucose concentrations (P<0.01) but both 10<sup>-6</sup>M and 10<sup>-11</sup>M ghrelin decreased insulin secretion in comparison to the control group (P-value<0.05) (Figure 3).

**The evaluation of islet cells and CRI-D2 cells for apoptosis**

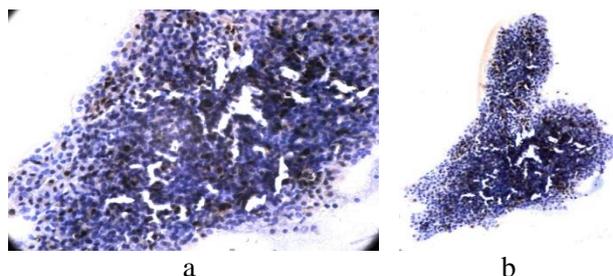
Not more than 5% of the cells treated with higher concentrations of ghrelin were apoptotic. No significant changes were observed in the cells treated with lower concentrations of ghrelin. Therefore, we concluded that this peptide had not been toxic to the cells (Figure 4, 5)



**Figure 3.** The effect of different concentrations of ghrelin on insulin secretion in presence of glucose. All data are shown in mean ± SD, Analysis by two-way ANOVA, \*P-value<0.05



**Figure 4. a)** The islets of Langerhans treated with 10<sup>-6</sup>M of ghrelin, b) The islets treated with 10<sup>-11</sup>M of ghrelin for 24 hr. Brown cells are apoptotic



**Figure 5. a)** CRI-D2 cells treated with 10<sup>-6</sup>M of ghrelin, b) CRI-D2 cells The treated with 10<sup>-11</sup>M of ghrelin for 24 hr. Brown cells are apoptotic

**Discussion**

Ghrelin has a key role in the control of metabolism and energy homeostasis (15). It is well established that ghrelin, as a metabolic signal, can modulate the function of the HPG axis, to a great extent, due to its ability to regulate GnRH neuronal function (16). There is some evidence to confirm that the modulatory effect of ghrelin on GnRH neurons is indirect and also mediated by regulating Kiss-1 expression in the hypothalamic area (17,18).

Besides, the role of ghrelin in the modulation of pancreatic function seems to be essential for regulation of glucose homeostasis and insulin secretion (19). But the mechanisms that the regulatory effect of ghrelin is mediated by have not been established. Similarly the ability of ghrelin to down-regulate Kiss1 expression in the hypothalamus may be a contributing factor in the ghrelin-related suppression of Kiss-1 expression and even insulin suppression in the pancreatic beta cell. We demonstrated that 10<sup>-6</sup>M of ghrelin is essential for suppression of Kiss-1 and KissR but lower concentrations are not effective. Our findings for the first time suggest the same inhibitory influence of ghrelin on Kiss-1 and KissR transcription in both islet cells and CRI-D2 cells. Therefore we suggest gene knock down and Western blotting assays to confirm these results.

Ghrelin has been shown to either stimulate or inhibit insulin secretion, depending on the experimental condition; Studies of isolated mouse islets showed that physiological concentrations of ghrelin (0.5-3 nmol) had no effect on glucose-stimulated insulin release, while low ghrelin concentrations (1-100 pmol) inhibited and high concentrations (0.1 and 1 micromol) stimulated insulin secretion (20). In another experiment, pancreatic tissue fragments of normal and diabetic rats were treated with different concentrations (10<sup>-12</sup>), 10<sup>-9</sup>), and 10<sup>-6</sup>) M) of ghrelin. Adeghate, *et al* revealed that insulin secretion was increased in both normal and diabetic rats after treatment with ghrelin (21). Furthermore, ablation of ghrelin and its receptor augmented insulin release and prevented impaired glucose tolerance in high-fat, diet-induced and leptin-deficient obese models (21, 22).

The existence of much controversies about the effect of ghrelin on insulin release, persuade us to set an

*in vitro* experiment on rat islets of Langerhans. We repeated the test in an insulinoma cell line (CRI-D2 cells) for further allegation. Our data evidently confirmed that both of higher and lower concentrations of ghrelin (10(-6) and 10(-11)) reduced insulin release in isolated rat islets. Similar inhibitory effects attained from investigations in isolated human and rat islets (12) and immortalized  $\beta$ -cell lines (13).

Therefore our results show the presence of both a mediator effect of ghrelin on Kiss-1 and KissR transcription and an inhibitory effect of ghrelin on insulin secretion in the islets of Langerhans.

Thus, it seems that the inhibitory effect of ghrelin on insulin secretion may be mediated by kiss-1. Therefore, a better understanding of its function and its suppressing pathway might be a potential therapeutic target for type 2 diabetes.

### Conclusion

Our findings suggest that the effect of ghrelin on insulin secretion in the islet cells probably mediated by Kiss-1 transcription pathway, although more investigations such as gene knock down and Western blotting should be done on the pancreatic beta cells to confirm our data.

In addition, our study confirms the inhibitory effect of ghrelin on insulin secretion in the rat isolated islets and CRI-D2 cells.

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### References

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999; 402:656-660.
2. Davenport AP, Bonner TI, Foord S M, Harmar, AJ, Neubig RR, Pin, JP, *et al.* International Union of Pharmacology. LVI. Ghrelin receptor nomenclature, distribution, and function. *Pharmacol Rev* 2005; 57:541-546.
3. Stengel A, Tache Y. Ghrelin a pleiotropic hormone secreted from endocrine X/A-like cells of the stomach. *Front Neurosci.* 2012;6:24.
4. Banks WA, Tschop, M, Robinson SM, Heiman ML. Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther* 2002; 302:822-827.
5. Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin, DG. Central nervous system control of food intake. *Nature* 2000; 404:661-671.
6. Forbes S, Li XF, Kinsey-Jones J, O'Byrne K. Effects of ghrelin on Kisspeptin mRNA expression in the hypothalamic medial preoptic area and pulsatile luteinising hormone secretion in the female rat. *Neurosci Lett* 2009; 28:143-147.
7. Fernandez-Fernandez R, Navarro VM, Castellano JM, Dieguez C, Aguilar E, Pinilla L, *et al.* Novel signals for the integration of energy balance and reproduction. *Mol Cell Endocrinol* 2006; 254:127-132.
8. Dungan HM, Clifton DK, Strainer R. Minireview: Kisspeptin Neurons as Central Processors in the Regulation of Gonadotropin-Releasing Hormone Secretion. *Endocrinology* 2006; 147:1154-1158.
9. Mead EJ, Maguire JJ, Kuc RE, Davenport AP, Kisspeptins: a multifunctional peptide system with a role in reproduction, cancer and the cardiovascular system. *Br J Pharmacol* 2007; 151:1143-1153.
10. Lely AJ, Tschop M, Heiman ML and Ghigo E. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocrine Reviews* 2004; 25:426-457.
11. Dezaki K, Hosoda H, Kakei M, Hashiguchi S, Watanabe M, Kangawa K *et al.* Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca<sup>2+</sup> signaling in beta-cells: implication in the glycemic control in rodents. *Diabetes* 2004; 53:3142-3151.
12. Colombo M, Gregersen S, Xiao J, Hermansen K. Effects of ghrelin and other neuropeptides (CART, MCH, orexin A and B, and GLP-1) on the release of insulin from isolated rat islets. *Pancreas* 2003; 27:161-166.
13. Wierup N, Yang S, McEvelly RJ, Mulder H, Sundler F. Ghrelin is expressed in a novel endocrine cell type in developing rat islets and inhibits insulin secretion from INS-1 (832/13) cells. *J Histochem Cytochem.* 2004; 52:301-310.
14. Date Y, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, Kojima M, *et al.* Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. *Diabetes* 2002; 51:124-129.
15. Cummings DE, Overduin J. Gastrointestinal regulation of food intake. *J Clin Invest* 2007; 117:13-23.
16. M T-S. Roles of ghrelin and leptin in the control of reproductive function. *Neuroendocrinology* 2007; 86:229-241.
17. Martini AC, Tovar S, *et al* Comparative analysis of the effects of ghrelin and unacylated ghrelin on luteinizing hormone secretion in male rats. *Endocrinology* 2006; 147:2374-2382.
18. Tena-Sempere M. Interaction Between Energy Homeostasis and Reproduction: Central Effects of Leptin and Ghrelin on the Reproductive Axis. *Horm Metab Res* 2013; 45:919-927.
19. Sun Y AM, Smith RG. Central and peripheral roles of ghrelin on glucose homeostasis. *Neuroendocrinology* 2007; 86:215-227.
20. Salehi A, Dornonville de la Cour C, Håkanson R, Lundquist, Effects of ghrelin on insulin and glucagon secretion: a study of isolated pancreatic islets and intact mice. *Regul Pept* 2004; 118:143-150.
21. Adeghate E, Ponery AS. Ghrelin stimulates insulin secretion from the pancreas of normal and diabetic rats. *J Neuroendocrinol* 2002; 14:555-560.
22. Ma X, Lin Y, Lin L, Qin G, Pereira FA, Haymond MW, *et al.* Ablation of ghrelin receptor in leptin-deficient ob/ob mice has paradoxical effects on glucose homeostasis when compared with ablation of ghrelin in ob/ob mice. *Am J Physiol Endocrinol Metab* 2012;303:E422-31.