*Praca oryginalna* *Original paper*

# Kisspeptin-10 and peptide 234 modulate GnRH--induced follicle-stimulating hormone secretion from anterior pituitary cells of prepubertal lambs in vitro*)

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## Kisspeptin-10 and peptide 234 modulate GnRH-induced follicle-stimulating hormone secretion from anterior pituitary cells of prepubertal lambs in vitro

Summary

**The aim of the study was to analyze the contribution of kisspeptin-10 (KiSS-10) and peptide 234 (kisspeptin-234, potent neutral antagonist of GPR-54 receptors) to the modulation of GnRH-induced follicle-stimulating hormone (FSH) secretion from anterior pituitary cells of prepubertal ram lambs in vitro. Pituitary cells were cultured in McCoy 5A medium without hormones (the negative control), with GnRH ($4 \times 10^{-9}$ M, the positive control), with GnRH ($4 \times 10^{-9}$ M) and $10^{-11}$-$10^{-8}$ M of KiSS-10 or GnRH ($4 \times 10^{-9}$ M), $10^{-11}$-$10^{-8}$ M of KiSS-10 and $10^{-7}$ M of peptide 234. After 6, 12 and 48 h of the experiment, the secretion of follicle-stimulating hormone was determined. The obtained results show that FSH secretion from anterior pituitary cells of ram lambs in vitro was dependent on kisspeptin-10 concentration in the culture medium. Addition of $10^{-11}$-$10^{-9}$ M of KiSS-10 caused an increase in FSH secretion (r = 0.73, 0.90, and 0.82 after 6, 12 and 48 h, respectively) compared to both the negative and positive control, whereas the highest concentration of KiSS-10 ($10^{-8}$ M) suppressed the secretion of this gonadotropin. The most stimulating effect was observed under the influence of $10^{-9}$ M of KiSS-10. However, concurrent cell exposure to peptide 234 abolished the stimulating action of kisspeptin-10 on FSH secretion. The negative correlation between FSH secretion and $10^{-11}$-$10^{-8}$ M of KiSS-10 in this condition was found (r = –0.68, –0.91, and –0.81 after 6, 12 and 48 h, respectively). This confirms that the observed increase in GnRH-induced FSH secretion was a direct effect of KiSS-10 on the anterior pituitary cells of prepubertal ram lambs.**

**Keywords: kisspeptin-10, peptide 234, follicle-stimulating hormone, anterior pituitary cells**

Kisspeptins, encoded by the kiss-1 gene, belong to the RF-amide peptide family and bind to the G-protein coupled receptor (GPR-54). It is known that the kisspeptin/GPR-54 system is involved in the hormonal cascade of reproductive cyclicity and in the regulation of gonadotropin secretion in many species. Moreover, kisspeptins are essential factors that induce the onset of puberty by the activation and progression of hypothalamic-pituitary-gonadal (HPG) axis (3, 8, 25). It is well established that the kisspeptin/GPR-54 system initiates the activity of GnRH neurons in the hypothalamus. As a result, GnRH is secreted into the hypophyseal portal circulation and induces the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary (1, 10). Then, LH stimulates the interstitial cells of the testes (Leydig cells) to produce testosterone, which has a permissive role for sperm production and maturation (7). FSH exerts influence on Sertoli cells and promotes spermatogenesis. Follicle-stimulating hormone also influences testicular development and plays a pivotal role in the maturation of the seminiferous tubule, proliferation of Sertoli cells, and production of germinal

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cells (12, 21, 28). However, there is no data on the direct effect of kisspeptin on FSH secretion in immature male sheep. Therefore, the aim of this study was to analyze the contribution of kisspeptin-10 (KiSS-10) and peptide 234 (potent neutral antagonist of GPR-54 receptors) to the modulation of GnRH-induced follicle-stimulating hormone secretion from anterior pituitary cells of prepubertal ram lambs *in vitro*.

## Material and methods

The protocol of the study and all procedures were approved by the Second Local Ethics Committee for Animal Experimentation in Lublin.

Pituitary glands were obtained from six-month-old Polish Lowland ram lambs (n = 6) immediately after slaughter. The present study was carried out on three independent cell cultures. Each cell culture was prepared using pituitaries isolated from two lambs. The anterior pituitaries were separated from the other lobes and washed twice with phosphate buffered saline (PBS). The tissue was minced into pieces of about 1 mm on each side, transferred to a sterile beaker containing 0.25% trypsin solution, and incubated at 37°C with stirring. Every 10 minutes, the suspension of dispersed pituitary cells was transferred to a conical centrifuge tube, supplemented with Dulbecco's Modified Eagle's Medium (DMEM) containing 0.1% BSA, 0.08% glucose, 0.59% HEPES, and gentamycin (20 µg/ml), and centrifuged at 1200 rpm for 10 min. The supernatant was discarded, and the cells were washed twice with DMEM. This procedure was repeated until the complete digestion of the pituitary fragments. The obtained cells were centrifuged (1200 rpm, 10 min), washed twice with PBS, and resuspended in the McCoy 5A medium containing 2.5% fetal calf serum, 10% horse serum, mixture of amino acids and vitamins, 0.59% HEPES, and gentamicin (20 µg/ml), and adjusted to pH 7.4 (4, 13-16). Next, the cells were stirred to obtain a homogenous suspension and counted with a haemocytometer. Cell viability was assessed by the trypan-blue-dye (0.4%) exclusion method. Afterwards, the McCoy 5A medium was added to achieve a final cell count of $2.5 \times 10^5$ live cells/ml. One milliliter of the prepared cell suspension was transferred to each well of 24-well culture plates and incubated at 37°C under the atmosphere of 5% $CO_2$ and 95% air. After the cells completely attached to the dishes and reached confluency (after 72 h of culture), the experiment was started. The cells were washed and then cultured in the serum-free McCoy 5A medium without hormones (the negative control), with GnRH ($4 \times 10^{-9}$ M; the positive control), with GnRH ($4 \times 10^{-9}$ M) and $10^{-11}$-$10^{-8}$ M of kisspeptin-10, and with GnRH ($4 \times 10^{-9}$ M), kisspeptin-10 ($10^{-11}$-$10^{-8}$ M) and peptide 234 (kisspeptin-234, a potent neutral antagonist of GPR-54 receptors, $10^{-7}$ M). Each sample was performed in duplicate. After 6, 12, and 48 h of the experiment, the media for FSH analysis were collected, and the proliferation index (PI) of the control cells and of those treated with KiSS-10 or KiSS-10 and peptide 234 was determined. The assessment of cell proliferation was based on the reduction of tetrazolium salt (MTT) to blue formazan. All cultures were pulsed with 15 µL of MTT (for 3 h at 37°C) and then solubilized with a 10% solution of sodium dodecyl sulphate (SDS) overnight. The optical density (OD) of the obtained blue formazan was measured with an ELISA microplate reader at a wavelength of 600 nm. The results were expressed as PI values and used to calculate FSH secretion. FSH concentration in the culture medium was determined with a Sheep Follicle-Stimulating Hormone ELISA Kit (Abnova Corporation, USA). FSH secretion was expressed as a concentration (ng/ml) of the hormone that was released into the culture medium by about $2.5 \times 10^5$ cells during 6, 12, or 48 hours of the experiment.

The obtained data were calculated by Statistica 5.0 PL and presented as the mean and standard deviation ($x \pm SD$). The comparisons between the control and experimental cultures were performed by analysis of variance and the paired *t*-tests. Differences were considered as significant at $P \leq 0.05$.

## Results and discussion

### The effect of kisspeptin-10 on GnRH-induced FSH secretion from anterior pituitary cells of prepubertal ram lambs *in vitro*.

Follicle-stimulating hormone secretion ranged from $0.94 \pm 0.14$ to $1.48 \pm 0.23$ ng/ml/$2.5 \times 10^5$ cells/6 h in the negative control culture and from $4.25 \pm 0.11$ ng/ml/$2.5 \times 10^5$ cells/6 h to $5.23 \pm 0.26$ ng/ml/$2.5 \times 10^5$ cells/48 h in the positive control culture. The influence of KiSS-10 on FSH secretion was dependent on the duration of exposure and the dose of kisspeptin used. The treatment of the cells with $10^{-11}$-$10^{-9}$ M of KiSS-10 resulted in increased FSH secretion. Under these conditions, follicle-stimulating hormone secretion reached the highest values after 48 h of experiment ($6.11 \pm 0.28$ ng/ml/$2.5 \times 10^5$ cells/48 h, $6.23 \pm 0.21$ ng/ml/$2.5 \times 10^5$ cells/48 h, $6.97 \pm 0.21$ ng/ml/$2.5 \times 10^5$ cells/48 h, respectively) (Fig. 1). In the presence of $10^{-9}$ M of KiSS-10, a significant ($P \leq 0.05$) increase in FSH secretion was found throughout the experiment ($5.53 \pm 0.10$ ng/ml/$2.5 \times 10^5$ cells/6 h, $6.70 \pm 0.13$ ng/

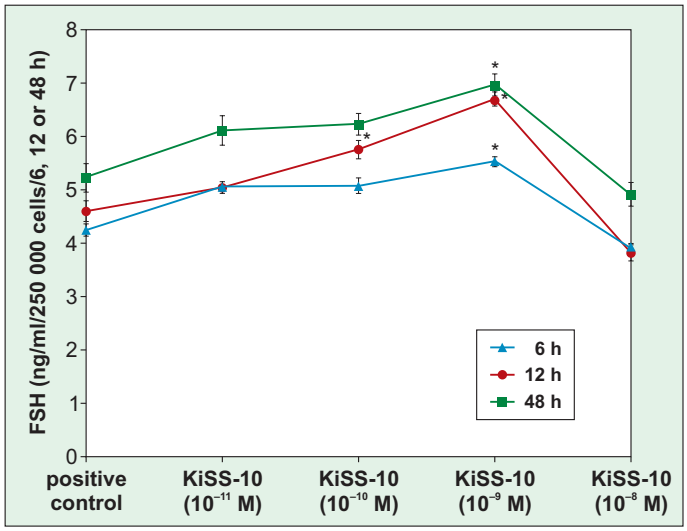


**Fig. 1. The influence of KiSS-10 (10-11 - 10-8 M) on GnRH-induced FSH secretion from the anterior pituitary cells of prepubertal ram lambs *in vitro***

Explanation: * – significant difference compared to the positive control ($P \leq 0.05$)

ml/2.5 × $10^5$ cells/12 h, 6.97 ± 0.21 ng/ml/2.5 × $10^5$ cells/48 h). However, $10^{-8}$ M of KiSS-10 caused the suppression of follicle-stimulating hormone secretion (3.92 ± 0.08 ng/ml/2.5 × $10^5$ cells/6 h, 3.82 ± 0.15 ng/ml/2.5 × $10^5$ cells/12 h, 4.92 ± 0.23 ng/ml/2.5 × $10^5$ cells/48 h), compared to the positive control (4.25 ± 0.11 ng/ml/2.5 × $10^5$ cells/6 h, 4.60 ± 0.19 ng/ml/2.5 × $10^5$ cells/12 h, 5.23 ± 0.26 ng/ml/2.5 × $10^5$ cells/48 h) (Fig. 1). The secretion of FSH from pituitary cells under the influence of $10^{-11}$-$10^{-9}$ M of kisspeptin-10 showed a very high positive linear correlation with KiSS-10 concentration (r = 0.73, r = 0.90, and r = 0.82 after 6, 12, and 48 h, respectively). However, taking into account also the highest concentration of KiSS-10, a negative correlation between FSH secretion and the kisspeptin dose ($10^{-11}$-$10^{-8}$ M) was found (r = –0.66, r = –0.63, and r = –0.60 after 6, 12, and 48 h, respectively).

**The effect of kisspeptin-10 and peptide 234 on GnRH-induced FSH secretion from pituitary cells of prepubertal ram lambs *in vitro*.**

The treatment of pituitary cells with peptide 234 ($10^{-7}$ M) resulted in the suppression of kisspeptin-modulated FSH secretion. The addition of both $10^{-11}$ M of KiSS-10 and peptide 234 decreased follicle-stimulating hormone secretion after 12 and 48 h, compared to the cultures with kisspeptin only. However, kisspeptin-10 in doses of $10^{-10}$-$10^{-8}$ M together with peptide 234 caused a reduction in FSH secretion during the whole experiment (Fig. 2 A, B, C). A significant difference ($P \leq 0.05$) between the experimental and positive control cultures was found only for peptide 234 and the highest KiSS-10 concentration after 12 and 48 h (3.37 ± 0.16 ng/ml/2.5 × $10^5$ cells/12 h *vs.* 4.60 ± 0.19 ng/ml/2.5 × $10^5$ cells/12 h, 4.24 ± 0.18 ng/ml/2.5 × $10^5$ cells/48 h *vs.* 5.23 ± 0.26 ng/ml/2.5 × $10^5$ cells/48 h, respectively) (Fig. 2 B, C). Thus, after the exposure of cells to peptide 234, negative correlations were found between kisspeptin-10 concentration ($10^{-11}$-$10^{-8}$ M) and FSH secretion (r = –0.68, r = –0.91 and r = –0.81 after 6, 12 and 48 h, respectively).

Kisspeptins play a crucial role in the process of puberty by initiating the activity of the hypothalamic-pituitary-gonadal (HPG) axis (26). It was noted that the expression of KiSS-1 and GPR-54 mRNA is elevated in the hypothalamus and testes of mammals at a prepubertal age, which suggests a direct impact of kisspeptin on the maturation of the male reproductive axis (22). KiSS-10 signaling in neurons of the hypothalamus, vicariously, via GnRH, stimulates gonadotropin secretion from the anterior pituitary cells (8, 17, 27). Afterwards, follicle-stimulating hormone promotes normal testicular development and spermatogenesis, as well as testosterone secretion (11, 12, 21, 29). It is well established that the pulsatile administration of kisspeptin induces gonadal activity, leading to the enhancement of ovarian steroidogenesis, stimulation of LH preovulatory surge, and initiation of cyclicity in prepubertal ewe lambs (23). Some reports indicate that kisspeptin may also prevent delayed puberty in female sheep (5). This disorder was found in ram lambs as well (2). It is observed in sheep undernourished during the

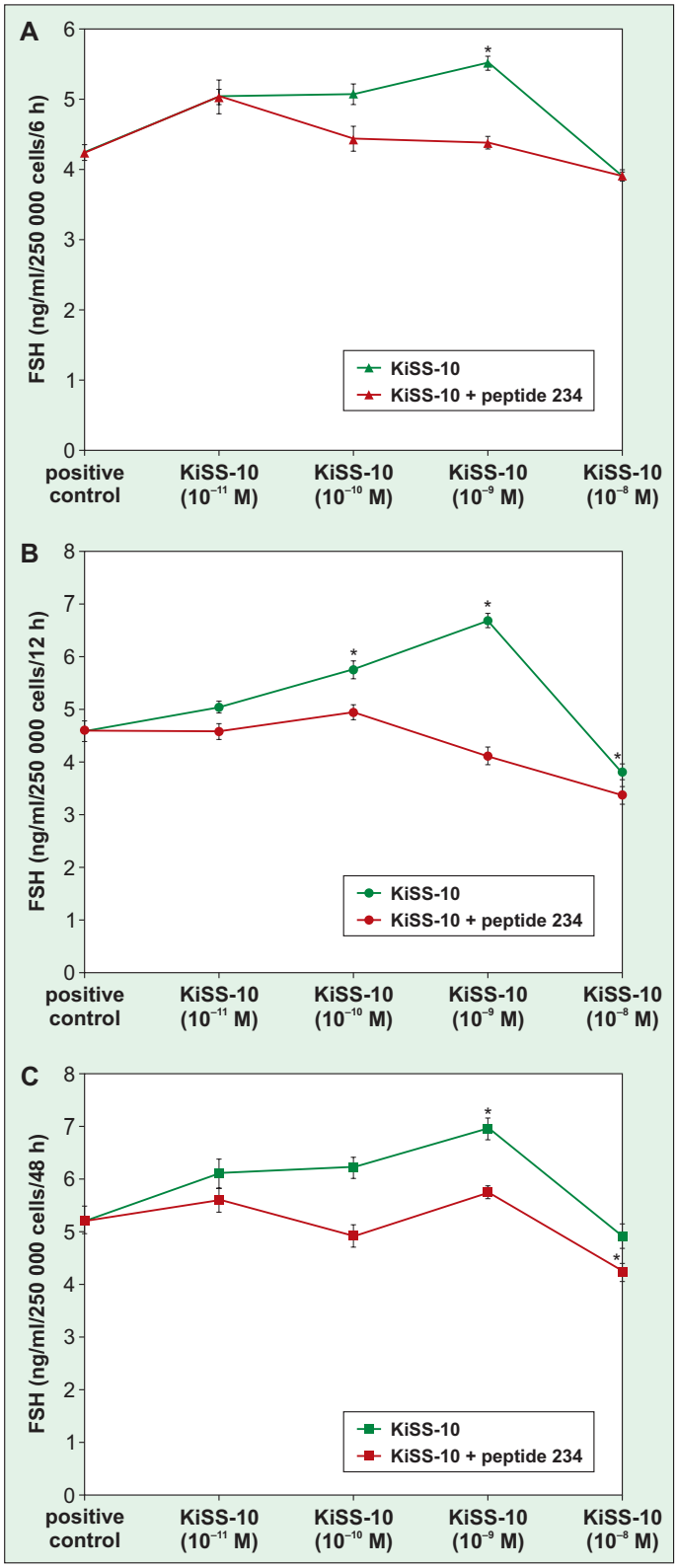


**Fig. 2. GnRH-induced FSH secretion from the anterior pituitary cells of prepubertal ram lambs *in vitro* under the influence of KiSS-10 ($10^{-11}$-$10^{-8}$ M) or KiSS-10 and peptide 234 ($10^{-7}$ M) after 6 h (A), 12 h (B) or 48 h (C)**

Explanation: * – significant difference compared to the positive control ($P \leq 0.05$)

early postnatal life or afflicted with hyperthyroidism (associated with a decrease in LH pulses and the inhibition of testicular growth) (6, 9). However, there are no available reports on a direct impact of kisspeptin on FSH secretion in immature male sheep. Therefore, the aim of our study was to determine the effect of kisspeptin on GnRH-induced FSH secretion from pituitary cells of prepubertal ram lambs.

The obtained results show that the secretion of follicle-stimulating hormone is dependent on kisspeptin concentration. The addition of $10^{-11}$-$10^{-9}$ M of KiSS-10 caused an increase in GnRH-induced FSH secretion compared to the positive control. This effect was confirmed by the positive correlation between KiSS-10 ($10^{-11}$-$10^{-9}$ M) and FSH secretion. Our results are consistent with data provided by Navarro et al. (20), which indicate that kisspeptin enhances GnRH-induced FSH secretion in male rats. There are also reports showing that both the central and peripheral infusion of the active kisspeptin fragment can stimulate the pituitary gland to increase the plasma concentration of FSH in male rodents *in vivo* (19, 25). However, some data also show that the chronic intraperitoneal administration of high doses of KiSS-10 causes a decline in gonadotropin secretion as well as testicular degeneration in prepubertal male rats (22). In our study, as well, the suppression of FSH secretion under the influence of the highest dose of kisspeptin ($10^{-8}$ M) was observed. This effect might be caused by the down-regulation of GPR-54 expression.

Peptide 234 is a potent neutral antagonist of GPR-54 receptor activity, which competes directly at the KiSS-10 binding site. It has a high binding affinity, specificity, and efficacy, as well as ability to inhibit KiSS-10 stimulation of inositol phosphate production in GPR-54-expressing cells (18, 24). It has previously been shown that the introduction of peptide 234 abolishes the action of kisspeptins both *in vivo* and *in vitro*. According to Roseweir et al. (24), intracerebroventricular (*i.c.v.*) administration of peptide 234 caused the suppression of KiSS-10-induced LH secretion in male rodents, but did not affect the basal luteinizing hormone release. It is also known that peptide 234 inhibits a castration-induced increase in LH in male rodents and sheep (18, 24). To date, there are no similar data on changes in FSH secretion from prepubertal ovine pituitary cells affected by KiSS-10 under normal and inhibited GPR-54. The present study shows that peptide 234 abolishes the stimulating effect of KiSS-10 on FSH secretion from pituitary cells of ram lambs. It suggests that the increase in follicle-stimulating hormone release was caused by a direct action of kisspeptin. The obtained results demonstrate that the concurrent exposure of cells to KiSS-10 ($10^{-11}$-$10^{-8}$ M) and peptide 234 causes a drop in follicle-stimulating hormone secretion compared to the cultures with kisspeptin only. This effect was confirmed by the negative correlation between KiSS-10 ($10^{-11}$-$10^{-8}$ M) and FSH secretion from ovine pituitary cells in vitro, modulated by kisspeptin and peptide 234.

The obtained results show a direct contribution of kisspeptin to the regulation of GnRH-induced FSH secretion from anterior pituitary cells of prepubertal ram lambs *in vitro*. Compared with the positive control, kisspeptin in a concentration of $10^{-11}$-$10^{-9}$ M enhanced FSH secretion, but reduced it in a dose of $10^{-8}$ M. The introduction of kisspeptin receptor antagonist abolished the stimulating effect of KiSS-10 on GnRH-induced FSH secretion from pituitary cells. The data presented here suggest the possibility of preventing delayed puberty in ram lambs by exogenous kisspeptin administration.

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