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LEPTIN EFFECT ON NITRIC OXIDE AND GnRH-INDUCED FSH SECRETION FROM OVINE PITUITARY CELLS *IN VITRO*.

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The secretion of gonadotrophins from anterior pituitary cells can be modulated by leptin and signals originating from the immune system, among others, by nitric oxide (NO). There are some studies that have demonstrated a role for leptin and NO in the regulation of FSH in rodents, however, no similar data are available in regard to ewes. Therefore, the objective of the present study was to analyse the leptin effect on GnRH-induced FSH secretion from the ovine anterior pituitary cells *in vitro.* Additionally, the influence of leptin on NO release and its role in the GnRH and leptin–modulated secretion of FSH from pituitary gland of ewes was investigated. The obtained results show that the influence of leptin on FSH secretion is biphasic. Leptin in concentration 10^{-8} and 10^{-7} M/l significantly enhances, whereas 10^{-6} and 10^{-5} M/l of leptin suppresses FSH secretion from the pituitary cells in comparison to the control. The secretion of FSH and NO release under the influence of leptin are in very high positive correlation (r=0.77). The inhibition of NO synthesis with L-NAME, instead, disables leptin from the stimulation of FSH secretion.

Key words: *leptin, FSH, nitric oxide, L-NAME, pituitary cells in vitro, ewes*

INTRODUCTION

Leptin plays a significant role in the control of female reproduction. Plasma leptin concentration increases together with the quantity of adipose tissue - the main source of leptin, and it is especially high in a case of fatness (1, 2). The physiological rise in plasma leptin concentration in ewes, especially during the late-luteal and follicular phase, is contributive to an increase in the ovulation rate (3, 4). However, in many species, an inordinately high plasma leptin concentration is related to fertility disorders, among other things, to an inhibition

of ovulation and ovarian cyst (OC) development. It is known that the main etiological factor of OC is an inappropriate, usually too low, amplitude of the preovulatory LH surge. Apart from the suppression of LH secretion from the pituitary, the ovarian cyst development in ruminants may result from a deficiency of the LH receptor in the ovary (5). As a consequence, LH cannot affect the dominant follicle and induce the ovulation. The expression of the LH receptor in the ovary is dependent on FSH. For this reason a decrease in FSH secretion during the late follicular phase may cause LH resistance. Moreover, FSH is needed for the induction of the activity of P450 aromatase in the granulosa cells of large non-atretic follicles and, this way, can increase the 17β-oestradiol (E-2) synthesis (6, 7, 8, 9). A deficiency of E-2 causes a suppression of the GnRH-mediated preovulatory surge of LH. Therefore, a decrease in FSH secretion during the late follicular phase may disrupt the ovulation and induce the ovarian cyst formation. Whereas there are few studies that have demonstrated a contribution of leptin in the modulation of FSH in rodents, there are not any similar data in regard to ewes. Taking into account the fact that fatness with the concomitant hyperleptinemia predisposes ewes to the ovarian cyst development, the first aim of the present study was to estimate the leptin effect on FSH secretion from ovine pituitary cells *in vitro*.

The secretion of hormones from anterior pituitary cells can be modulated by signals originating from the immune system (10), among others, by nitric oxide (NO) (11-15). The precise role of NO in modulating gonadotropin secretion from the pituitary, remains largely unexplored. According to many authors (14, 16, 17), NO mediates GnRH and leptin – induced gonadotropin release from the pituitary gland in rats. However, there are also contrary reports. According to Chatterjee (18), NO suppresses GnRH-induced gonadotropin release and the inhibition of NOS facilitates gonadotropin, especially LH, secretion from rat pituitary. Whereas the data mentioned above have demonstrated a role for NO in the regulation of gonadotropins in rodents, no similar data are available in regard to ewes. Therefore, in the second part of this study we have examined the influence of leptin on NO release and its role in the GnRH and leptin–modulated secretion of FSH from the pituitary gland of ewes.

MATERIALS AND METHODS

Experiment 1. Pituitary glands were obtained from 3-4 year-old crossbreed ewes (50% Suffolk + 25% Romanov + 25% Polish Lowland Sheep) at slaughter. Isolation of cells was carried out through the digestion of the pituitary with 0.25 % trypsin solution. Pituitary cells were finally cultured in McCoy 5A medium containing 2.5% fetal calf serum, 10% horse serum, mixture of amino acids and vitamins, gentamicin (20 μg/ml), and adjusted to pH 7.4 (19, 20). 1 ml (in the case of FSH secretion analysis) or 100μl (in the case of proliferation index determination) of dispersed cell suspension at a concentration of 2.5×10^5/ml was transferred to each culture dish of 24-well (or 96-well, respectively) culture plates and incubated for 84 h at 37°C under the atmosphere of 5%

CO_2. After attachment to the dishes, the cells were washed with McCoy 5A medium without serum, and finally incubated with McCoy 5A without hormones (negative control), with GnRH (4×10^{-9} M) (positive control) or with GnRH (4×10^{-9} M) and 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} or 10^{-5} M/l of the recombinant ovine leptin (*ro* leptin), respectively. Each sample was performed in duplicate. After 2, 6, 12, 18, 24 and 30 h of incubation the media for FSH and NO analysis were collected and the proliferation index (PI) of control cells and those treated with leptin was determined. Assessment of cell proliferation was based on the reduction of the tetrazolium salt (MTT) into a blue formazan. Control cultures and those incubated with leptin were pulsed with 15 µl of MTT (for 3h at 37°C) and then solubilised with SDS overnight. The optical density (OD) of the formed blue formazan was measured by ELISA microplate reader at the wavelength of 600 nm. The results were expressed as PI values. FSH concentration in the culture medium was determined using FSH [^{125}I] IRMA KIT (Orion Diagnostica, Spectria, Finland). FSH secretion was expressed as a concentration (IU/l) of hormone which was released into the culture medium by about 2.5×10^4 gonadotrophs during 2, 6, 12, 18, 24 and 30 h, respectively. Aliquots from the experiment described above were used for measuring concentrations of nitrite (NO_2^-) as an indicator of nitric oxide (NO) production. Equal amounts of sample and Griess reagent (sulfanilamide 2% (w/v), N-(1-naphtyl)ethylenediamine 0.2% (w/v), phosphoric acid 4% (v/v)) were mixed. After 10 minutes of incubation at room temperature the absorbance at 545 nm was measured. As a standard $NaNO_2$ was used (19). Each sample was performed in duplicate.

Experiment 2. Pituitary cells were incubated with McCoy 5A medium without hormones (negative control), with GnRH (4×10^{-9} M) (positive control) or with GnRH (4×10^{-9} M) and 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} or 10^{-5} M/l of the recombinant ovine leptin, respectively. All cultures were divided into two groups and incubated with or without N$^{\omega}$-Nitro-L-arginine methyl ester (L-NAME, 3×10^{-4} M). After 2, 6, 12, 18, 24 and 30 h the media for LH and NO analysis were collected. Each sample was performed in duplicate.

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

RESULTS

The effect of leptin on FSH secretion by ovine pituitary cells in vitro. Basal FSH secretion averaged 0.94 ± 0.08 IU/ml/ 2.5×10^4 gonadotrophs/ 2 h. The effect of leptin on FSH secretion by ovine pituitary cells was dependent on the dose of leptin used. The addition of 10^{-8} and 10^{-7} M/l leptin to the culture medium resulted in a significant ($P \leq 0.05$) increment in FSH secretion during the whole period of experiment with maximum values 4.01 ± 0.07 and 3.79 ± 0.09 IU/ml/2.5×10^4 gonadotrophs after 30 h, respectively. On the contrary, pituitary cells incubated with higher doses of leptin (10^{-6} and 10^{-5} M/l) decreased amounts of secreted FSH (*Fig.1*). Treatment with 10^{-10} and 10^{-9} M/l of leptin did not affect FSH secretion significantly (*Tab. 1*). Mean FSH secretion (IU/ml/25000gonadotrophs/1h) during the experiment was also dependent on the dose of leptin. It was increased during incubation of cells with leptin in concentration 10^{-10} – 10^{-7} M/l and reached the highest value (0.134 ± 0.007 IU/ml/25000gonadotrophs/1h) under the influence of 10^{-8} M/l of leptin. Leptin in the highest of the used doses: 10^{-6} and 10^{-5} M/l, instead,

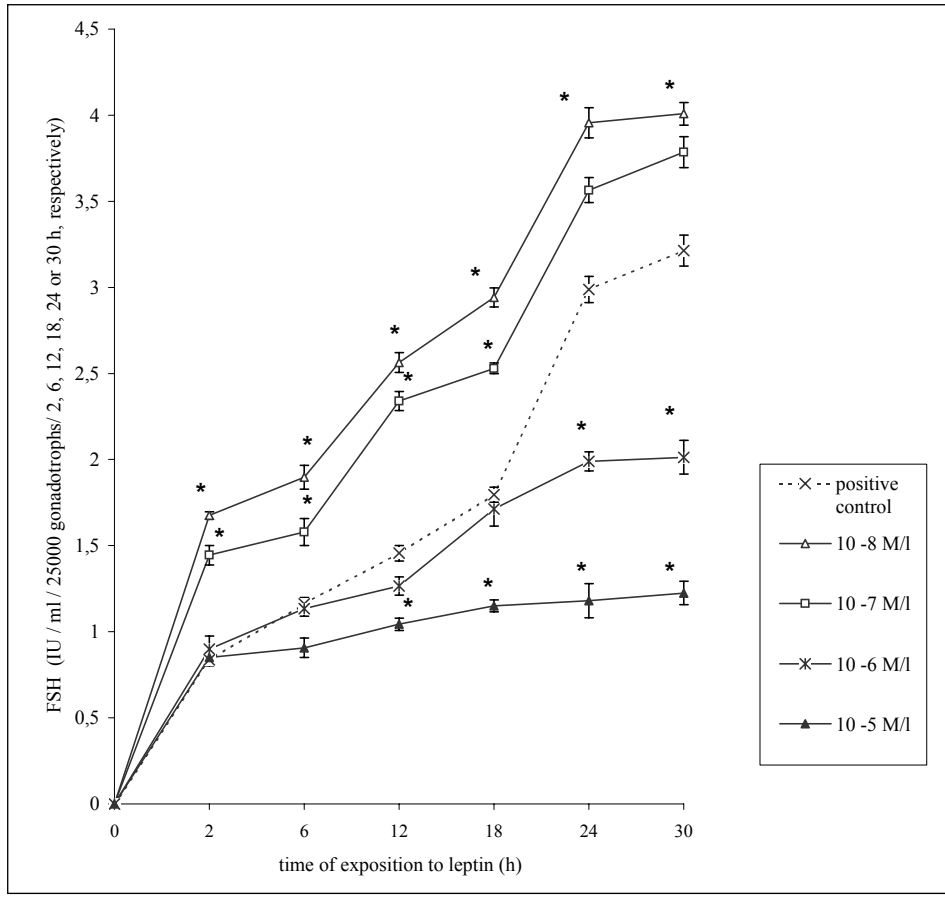

Fig. 1. The influence of leptin on GnRH-induced FSH secretion from ovine pituitary cells *in vitro.*
* - significant (P≤0.05) difference in comparison to control

Tab. 1. FSH secretion by ovine pituitary cells *in vitro* under the influence of leptin in concentration 10^{-10} and 10^{-9} M/l.

leptin concentration in culture medium (M/l)	**FSH secretion by ovine pituitary cells *in vitro* (IU/ml/2.5 × 10^4 cells)**					
	time of exposition of cells to leptin (h)					
	2	**6**	**12**	**18**	**24**	**30**
0 (control)	0,94±0,06	1,16±0,08	1,46±0,16	1,79±0,19	2,98±0,17	3,21±0,29
10^{-10}	0,96±0,07	1,20±0,14	1,57±0,15	1,89±0,19	3,09±0,21	3,35±0,19
10^{-9}	1,04±0,16	1,23±0,16	1,69±0,23	1,98±0,14	3,37±0,19	3,46±0,23

caused the significant (P≤0.05) drop in mean FSH secretion (0.067 ± 0.014 and 0.040 ± 0.006 IU/ml/25000gonadotrophs/1h) in comparison to the control (0.107 ± 0.009 IU/ml/25000gonadotrophs/1h) (*Fig.2*).

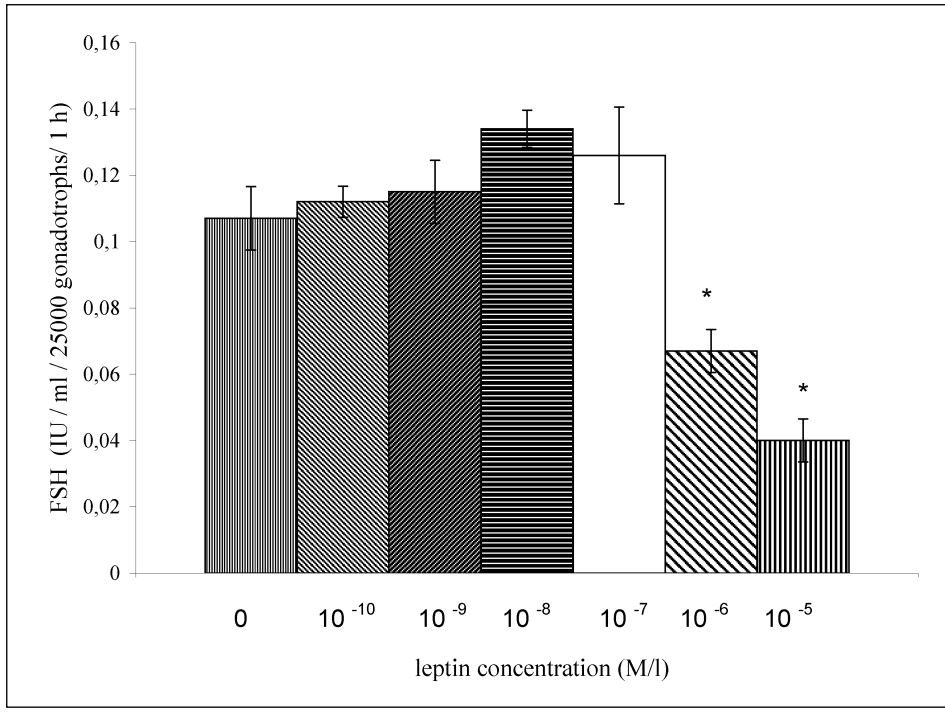

Fig. 2. The mean secretion of FSH (IU / ml / 25000 gonadotrophs/ 1 h) from ovine pituitary cells *in vitro* under the influence of the different concentrations of leptin.
* - significant (P≤0.05) difference in comparison to control

The effect of leptin on NO release by ovine pituitary cells in vitro. The influence of leptin on NO release was dependent on time and the dose of leptin used. The introduction of leptin in concentration of 10^{-8}, 10^{-7} and 10^{-6} M/l caused a significant (P≤0.05) augmentation in NO in the culture medium. 10^{-9} M/l leptin increased NO release significantly, but only after 2, 6, 12 and 30 h of incubation. Conversely, the highest of the leptin concentrations used (10^{-5} M/l) significantly (P≤0.05) reduced NO release. Leptin in the dose of 10^{-10} M/l did not affect NO release from ovine pituitary cells in vitro (*Fig.3*).

The relationship between FSH secretion and NO release by ovine pituitary cells under the influence of leptin. The secretion of FSH and NO release under the influence of leptin were in very high positive correlation (r=0.77) (*Fig. 4*). The almost full positive linear correlation between FSH and NO was found after using leptin in concentrations 10^{-8}, 10^{-7} and 10^{-5} M/l (r=0.96, 0,94 and 0.96, respectively).

The effect of leptin on FSH secretion by ovine pituitary cells in vitro in the presence of L-NAME. Inhibition of NO synthesis by the treatment of ovine pituitary cells with L-NAME, irrespective of the dose of leptin (10^{-10} – 10^{-5} M/l) used, disabled it from a stimulation of FSH secretion (*Fig.5*).

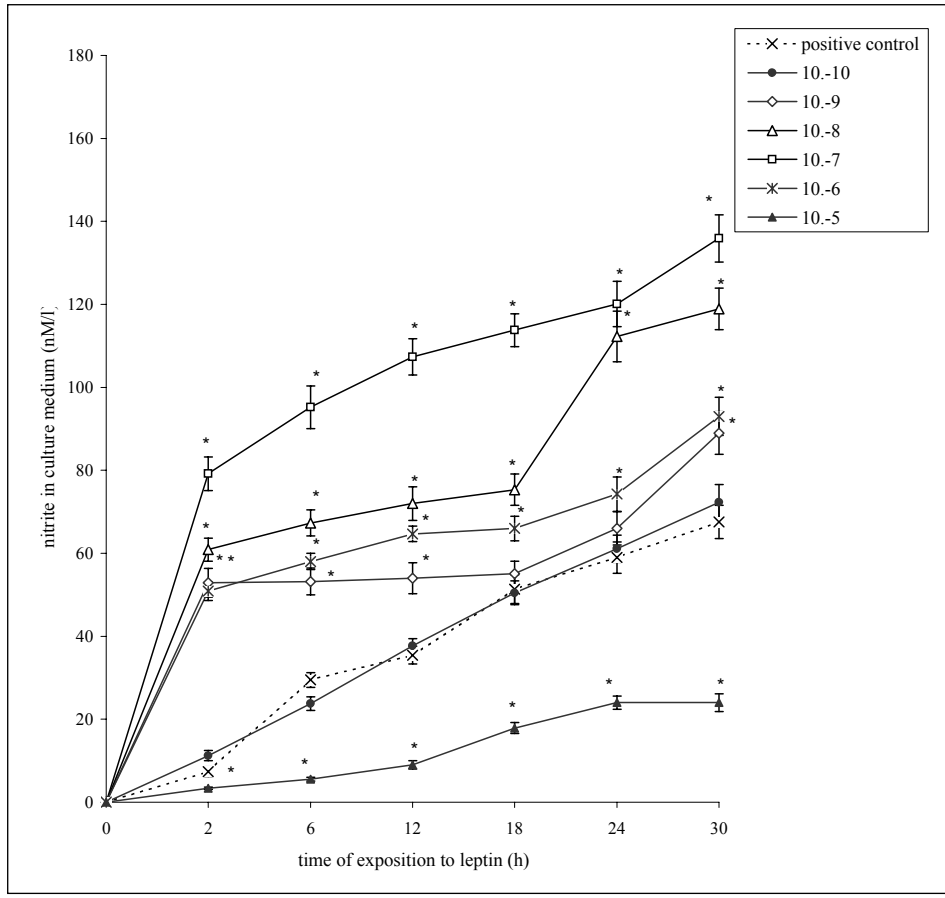

Fig. 3. Leptin effect on nitric oxide release from ovine pituitary cells *in vitro* in the presence of GnRH (4×10^{-9} M/l).
* - significant (P≤0.05) difference in comparison to control

The effect of leptin on the proliferation of ovine pituitary cells in vitro. The addition of leptin, irrespective of concentration, enhanced the proliferation of pituitary cells in vitro. Doses 10^{-9}, 10^{-8} and 10^{-7} M/l significantly (P≤0.05) increased proliferation in comparison to control (PI=1) during the whole experiment. After 30 h PI was the highest and reached 1.73 ± 0.05, 1.88 ± 0.09 and 1.58 ± 0.06, respectively at the doses mentioned above (control – PI=1). Incubation of cells with the lowest concentrations (10^{-10} M/l) resulted in a marked (P≤0.05) increase of proliferation activity only after 30 h (PI = 1.43 ± 0.09). A high positive linear correlation between the proliferation index of ovine pituitary cells and the used concentration of leptin was found (r=0.59) (*Fig.6*).

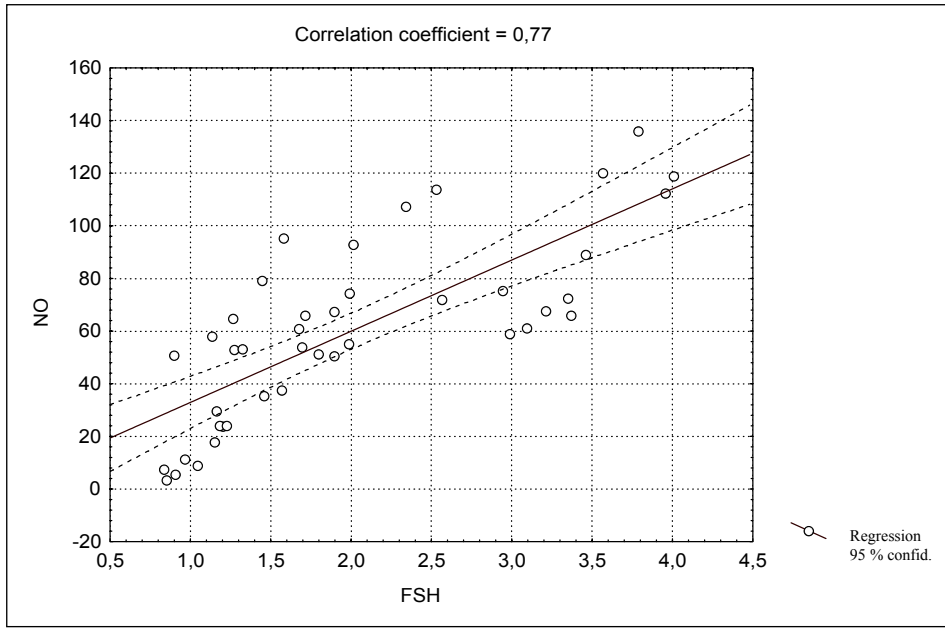

Fig. 4. The relationship between FSH secretion and NO release by ovine pituitary cells under the inluence of leptin.

DISCUSSION

There are some studies concerning leptin influence on GnRH-induced gonadotropins secretion, including NO contribution, in rats (17, 18, 21 - 23). However, their results are equivocal. Some authors (18, 21) have reported that NO reduces gonadotropins, especially LH release, whereas the inhibition of NOS, and thus the drop in NO release, causes an increment in gonadotropin secretion in response to GnRH. The results of others show that gonadotropin release is not inhibited, but stimulated by NO, especially when this stimulation is brought about by leptin or GnRH (17, 22, 23). Till now, no similar data on sheep have been produced. The results of our studies on ewes point out that leptin (in concentration 10^{-8} and 10^{-7} M/l) stimulates the GnRH-induced FSH secretion by ovine pituitary cells *in vitro* in a way dependent on the used leptin dose. It is significant that there is a similar relationship between leptin dose and changes in NO release and FSH secretion. So, there is also a high, very high or almost full positive correlation between changes in NO release and FSH secretion under the influence of the respective doses of leptin. Additionally, the present study reveals that the synthesis of NO by pituitary cells is necessary for the manifestation as well as maintenance of the positive effect of leptin on FSH secretion. This may be due to the fact that NO can activate guanylate cyclase and thus increase the synthesis of cGMP, which is responsible for the release of FSH (24 - 26). The

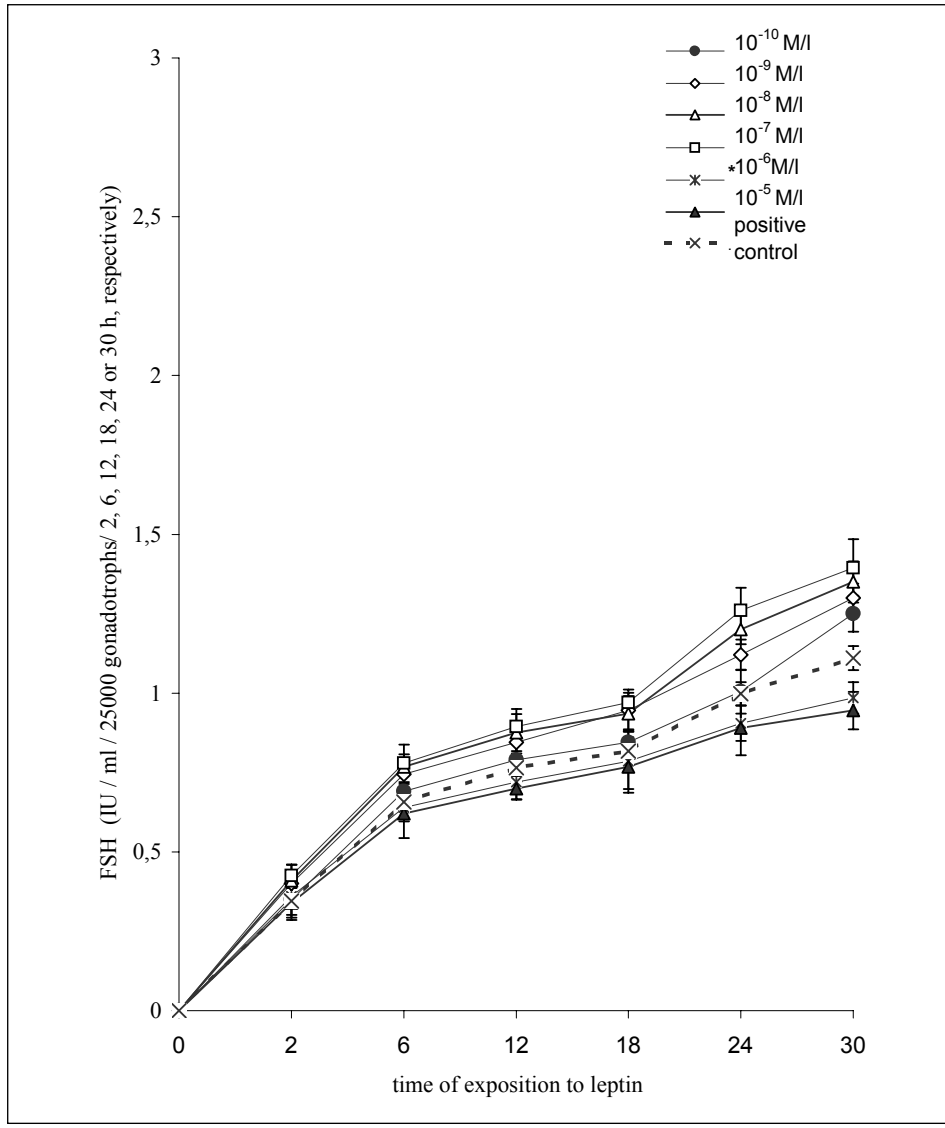

Fig. 5. The influence of leptin on GnRH-induced FSH secretion from ovine pituitary cells *in vitro* in the presence of L-NAME (3×10^{-4} M/l)

administration of NOS inhibitor - L-NAME prevents leptin from stimulating FSH secretion. In contrast to the results mentioned above, the highest doses of leptin (10^{-6} and 10^{-5} M/l) suppress FSH secretion. The possible reason for this occurrence may be the down-regulation of the leptin receptor in pituitary cells by leptin in this concentration.

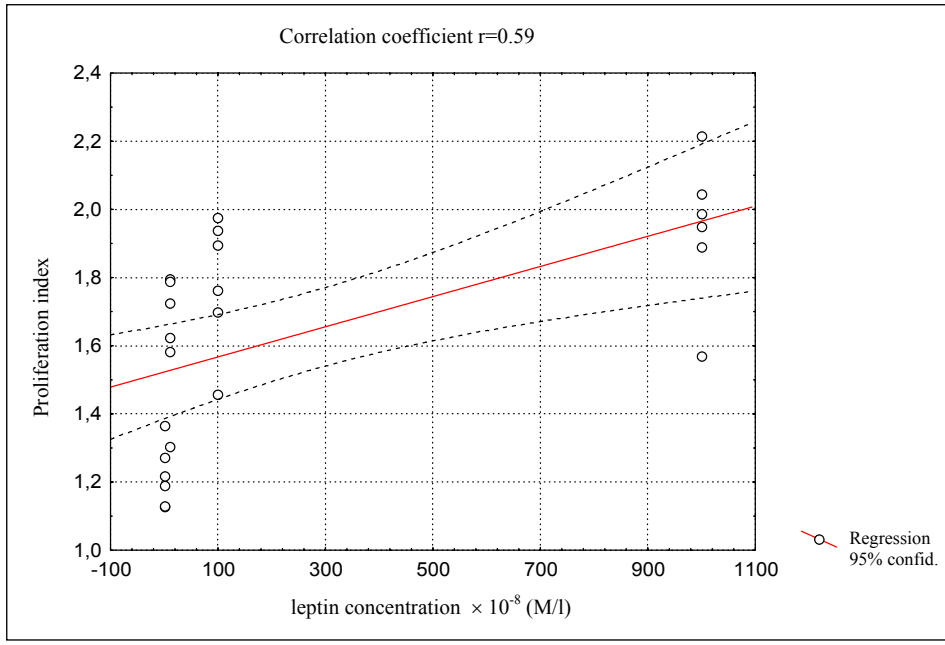

Fig. 6. The relationship between leptin concentration in culture medium and proliferation index of ovine pituitary cells *in vitro*.

Additionally, the obtained results show that leptin enhances the proliferation of ovine anterior pituitary cells *in vitro*. However, our findings are at variance with data obtained on human and rat pituitary cell lines by other authors (1, 27, 28). According to Jin and Morash (27, 28) leptin in concentrations 10^{-8} – 10^{-6} M inhibits pituitary cell proliferation in human and rat pituitaries *in vitro*. The reason of this difference may be due to fact that all previous experiments concerning leptin effect on pituitary cells proliferation were carried out on neoplastic cells, whereas in our study normal pituitary cells isolated from healthy sheep were used. There are, instead, a number of data showing the stimulatory influence of leptin on proliferation of normal mammalian cells isolated from other organs and tissues. Among other things, it is known that leptin has mitogenic effect on neurons of neocortex (29), glomelural endothelial cells (30), epithelial cells of mammary gland (31) and gastric mucosal cells (32).

The obtained results show that leptin in doses 10^{-8} – 10^{-7} M/l stimulates GnRH – induced FSH secretion from ovine pituitary cells *in vitro* and it appears that this effect, also in ewes, is mediated by nitric oxide. Additionally, leptin enhances the proliferation of normal ovine anterior pituitary cells *in vitro*.

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