

EFFECT OF GnRH AND ITS ANTAGONIST (ANTARELIX) ON LH RELEASE FROM CULTURED BOVINE ANTERIOR PITUITARY CELLS

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In the following investigations, the LH secretion of cells from pituitaries in heifers on days 16–18 of their oestrous cycle ($n = 14$) was analysed. Cells were dissociated with trypsin and collagenase and maintained in a static culture system. For the estimation of LH release, the cells were incubated with various concentrations of mammalian GnRH (Lutrelief) for 6 h. To determine the action of Antarelix (GnRH antagonist), the cells were preincubated for 1 h with concentrations of 10^{-5} or 10^{-4} M Antarelix followed by 10^{-6} M GnRH coincubation for a further 6 h. At the end of each incubation, the medium was collected for LH analysis. Parallel, intracellular LH was qualitatively detected by immunocytochemistry. Changes in the intensity of LH staining within the cells in dependence of different GnRH concentrations were not observed, but a significant increase LH secretion in pituitary cells was measured at 10^{-6} M GnRH. Antarelix had no effect on basal LH secretion at concentrations of 10^{-4} and 10^{-5} M. After coincubation of pituitary cells with Antarelix and GnRH, Antarelix blocked the GnRH-stimulated LH secretion with a maximal effect of 10^{-4} M, but the staining of immunoreactive intracellular LH was detected at approximately the same level compared to the pituitary cells treated with exogenous GnRH alone. These data demonstrate that Antarelix is effective in influencing the GnRH-stimulated LH secretion of pituitary cells *in vitro*. After administration of Antarelix *in vivo*, the GnRH-stimulated LH secretion of cultured pituitary cells was not inhibited.

Key words: Pituitary cells *in vitro*, GnRH and antagonist, LH, bovine

The decapeptide gonadotropin-releasing hormone (GnRH) plays a pivotal role in controlling the oestrous cycle in mammals. In contrast to GnRH agonists, treatment with antagonists like Antarelix (Deghenghi et al., 1993) inhibited gonadotropin secretion followed by a change in follicular growth and development (Driancourt et al., 1995; Campbell et al., 1997; Dobson et al., 1997; Fike et al., 1997; Patterson-Bay et al., 1997; Oussaid et al., 1999; Schneider et al., 1999;

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Brüssow et al., 2001). Antagonists to GnRH inhibit gonadotropin secretion, presumably by competing with GnRH for its receptor on the pituitary gonadotrope. Most studies examining the potency of GnRH antagonists have used *in vivo* tests. In bovine, the GnRH antagonist Antarelix manifests prolonged and reversible inhibition of pituitary gonadotropin secretion after multiple injections of each 1.5 mg at 12-h intervals (Schneider et al., unpublished data). Contrarily to Braun et al. (1985) who used non-identified material from the slaughterhouse, we exclusively used pituitaries from heifers on days 16–18 of the oestrous cycle. The reason was that the number of pituitary GnRH receptors varies during the cycle (Clayton et al., 1980) and is highest during the preovulatory period, shortly before the endogenous LH surge occurs (Nett et al., 1987).

In the present study, we have examined the influence of the GnRH antagonist Antarelix applied *in vitro* and *in vivo* on GnRH-mediated gonadotropin secretion by cultured bovine anterior pituitary cells. The release of FSH is regulated by additional factors, like pituitary and ovarian peptides and probably a specific releasing hormone (Padmanabhan and McNeilly, 2001); therefore, it seems to be impossible to prove antagonist effects under *in vivo* conditions identical for both gonadotropins. We analysed the LH release and immunohistochemical detection of LH in the pituitary after *in vivo* treatment with Antarelix compared to that of non-treatment.

Materials and methods

Animals

German Holstein heifers (n = 14) between 22 and 25 months of age with a body weight of 536 ± 27 kg were used. Prior to the experiments all animals had a normal oestrous cycle with a length of 21 days. The animals were slaughtered on days 16, 17, or 18 of a spontaneous cycle.

Pituitary tissue

Pituitary glands were collected immediately after slaughter. The posterior and intermediate lobes were removed and either the whole anterior pituitary was recovered for the cell preparation, or it was bisected for immunohistochemical detection of intracellular LH of cells in pituitary tissue.

Dispersion and culture of anterior pituitary cells

After transport on ice in medium 199 (HEPES modification; Sigma, Deisenhofen, Germany) + 7.5 mM glucose, the whole anterior pituitary was cut into small pieces in a Petri dish. The cut pieces were allowed to settle in a 50 mL sterile conical centrifuge tube containing Hank's Balanced Solution (HBSS; Sigma). The medium was decanted and replaced twice with fresh HBSS to re-

move lysed cells whose contents were released during mincing of the tissue. The pieces of tissue were enzymatically treated in the same manner as previously described for uterine cells (Tiemann et al., 1994). Aliquots of 1 mL or 200 μ L of the resuspended cells (1.5×10^5 cells/mL) were plated in 24-well plates or 8-well chamber slides (Nunc, Naperville, IL, USA), respectively. Viability was evaluated by trypan blue exclusion (90–95%); at the end of the experiments, cell viability of the adherent cells was further assessed in the same way and was 95%. The cells were incubated at 37 °C in 95% O₂/5% CO₂ for 2 days. The culture medium was then removed and the plated cells were rinsed twice with HBSS to remove non-adherent cells. Each well was filled with 1 mL or 200 μ L aliquot of medium 199 (10% FCS + 1% ABAM: 10,000 U/mL penicillin, 10 mg/mL streptomycin and 25 μ g/mL amphotericin, Sigma) for a further 48 h for the 24-well plate or 8-well chamber slide, respectively. Cells from each heifer were cultured separately. The cells were incubated at 37 °C in 95% O₂/ 5% CO₂ for 2 days.

GnRH and GnRH antagonist

The mammalian GnRH (Lutref, p-Glu His Trp Ser Tyr Gly Leu Arg Pro Gly-NH₂) was purchased from Ferring (Kiel, Germany). The GnRH antagonist (Antarelix, Ac-D-Nal D-Cpa D-Pal Ser Tyr D-Ha Leu Lys[iPr] Pro D-Ala-NH₂) was synthesised by Europeptides (Argenteuil, France).

Experimental protocol

Following the 96-h culture period the old medium was discarded and plates were rinsed twice with HBSS to remove serum and unattached cells. Each well of the 24-well plate or 8-well chamber slides was, respectively, filled with a 500 μ L or 200 μ L aliquot of Start V medium (Biochrom, Berlin, Germany) supplemented with 1% ABAM and preincubated for 1 h. Cells grown in 24-well plates were used for the estimation of LH release, whereas cells grown in 8-well chamber slides were used for characterisation of cell types in the cell culture and for qualitative detection of intracellular LH.

Experiment 1

To determine the effects of increasing GnRH concentrations on the LH release, the cells were incubated for 6 h with Start V medium (1% ABAM) supplemented with or without different concentrations (10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} M) of GnRH (n = 4). In order to determine the *in vitro* effect of Antarelix on the GnRH-stimulated LH secretion of pituitary cells, the cells were pretreated with or without Antarelix at concentrations of 10^{-5} , 10^{-4} M for 1 h followed by a coincubation for 6 h with or without 10^{-6} M GnRH (n = 6). The effect of Antarelix on basal LH secretion *in vitro* was investigated in pituitary cell cultures derived from six heifers.

The intracellular localisation of LH in pituitary cells grown in monolayer (chamber slides) after *in vitro* treatment with GnRH and/or Antarelix was detected using a polyclonal antibody against LH.

Experiment 2

In order to determine the effect of Antarelix after its administration *in vivo* on the GnRH-stimulated LH secretion of pituitary cells *in vitro*, two heifers on the oestrous cycle day 17 were injected intramuscularly with Antarelix (1.5 mg in 5 mL 5% mannitol/injection) a total of six times (at 8:00 h and 20:00 h for 3 days). After slaughtering, the pituitary cells were isolated and cultured as described above. The cells were incubated with or without GnRH ($0, 10^{-6}, 10^{-5}$ M) for 6 h.

After finishing experiments 1 and 2, the medium was collected for LH determination and stored at -20°C .

In order to investigate the intracellular localisation of LH in pituitary tissue after *in vivo* treatment with Antarelix compared to the control animals ($n = 2$), serial tissue sections were stained for LH.

LH estimation with ECLIA

The electrochemiluminescence immunoassay (ECLIA) was performed in the same manner as previously described (Schneider et al., 1999). This test system was developed according to Deaver (1995) and we used the monoclonal antibody 518 B7 against ovine LH (Matteri et al., 1987) for TAG-labelling [N-hydroxysuccinimide ester of a ruthenium (II) *tris*-bipyridine chelate] (IGEN, Inc. Gaithersburg, USA). Rabbit anti-bovine LH was used as first antibody and highly purified bovine LH (Biotrend, Köln, Germany) as standard. The bound/free separation was performed by addition of a second antibody (sheep anti-rabbit IgG) coupled to magnetic beads (Dynal, Hamburg, Germany). The measurement of luminescence signals was carried out by the ORIGEN 1.5 analyser (IGEN). The sensitivity of the method was calculated to be 0.03 ng/mL. The intra- and interassay CV were 6.4% and 8.9%, respectively.

Indirect immunofluorescence

To evaluate the cell types present in culture, pituitary cells were analysed by immunofluorescence using antibodies specific for epithelium (anticytokeratin) and fibroblasts (antivimentin) in the same manner as described previously (Tiemann et al., 1996). The DNA-binding dye, 33258 Hoechst, was added to the cells at a final concentration of 4 μM for 5 min at room temperature. After additional washes (three times), slides were mounted in glycerol containing *p*-phenylenediamine and analysed for fluorescence using a Nikon epifluorescence microscope. Immunofluorescence controls consisted of the secondary and fluorochrome conjugates without primary antibody.

Immunohisto(cyto)chemical staining for LH

Bovine pituitary tissues of Antarelix treated and untreated heifers were fixed in Bouin's fixative at room temperature for 7 h; they were then washed in 70% ethanol at room temperature overnight. After fixation, tissue was dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin for sectioning; 5 μ m sections were cut and mounted on poly-L-lysine slides. Briefly, sections were deparaffinised and rehydrated.

Adherent pituitary cells grown in chamber slides were incubated for 6 h with GnRH or preincubated with Antarelix for 1 h and followed with GnRH for a further 6 h. Cells were washed in Tris-buffered saline (TBS: 20 mM Tris, 500 mM NaCl, pH 7.5) and fixed with ice-cold methanol at -20°C for 10 min. After fixation, slides were air-dried and subsequently rehydrated with TBS, wrapped in parafilm, and stored at 4°C until use.

In tissue slides and adherent cells, localisation of LH was detected using a streptavidin-immunoperoxidase technique and performed as described previously (Tiemann et al., 2001a, b). In our immunohisto(cyto)chemical staining experiments we used, as the primary antibody, a rabbit polyclonal anti-LH antibody (Biotrend) at 1:4000 dilution for 24 h at 4°C . To test the specificity of the immunostaining: (1) antiserum was incubated with increasing concentrations of bovine LH (Biotrend) for 24 h at 4°C ; and (2) the primary antibody was substituted with nonimmune serum; and (3) the primary antibody was omitted.

Statistical analysis

In all experiments, treatments were tested in quadruple wells. Data are expressed as means \pm SEM. Treatments were analysed in multifactorial designs (ANOVA) which included the effect of treatments. The Fisher's robust LDS procedure supplied by SAS Release 6.08) was used to compare between group differences, significance level $\alpha = 0.05$.

Results

Characterisation of pituitary cells

Approximately 50% of pituitary cells dispersed enzymatically and grown to confluence on plastic reacted positively with antibody to cytokeratin, an epithelial cell marker. Similarly, about 50% of the cells reacted with an antibody to vimentin, an intermediate filament protein present in fibroblasts. Thus, the enzymatically dispersed cell cultures grown on plastic contained a mixture of epithelial and stromal cells (Fig. 1).

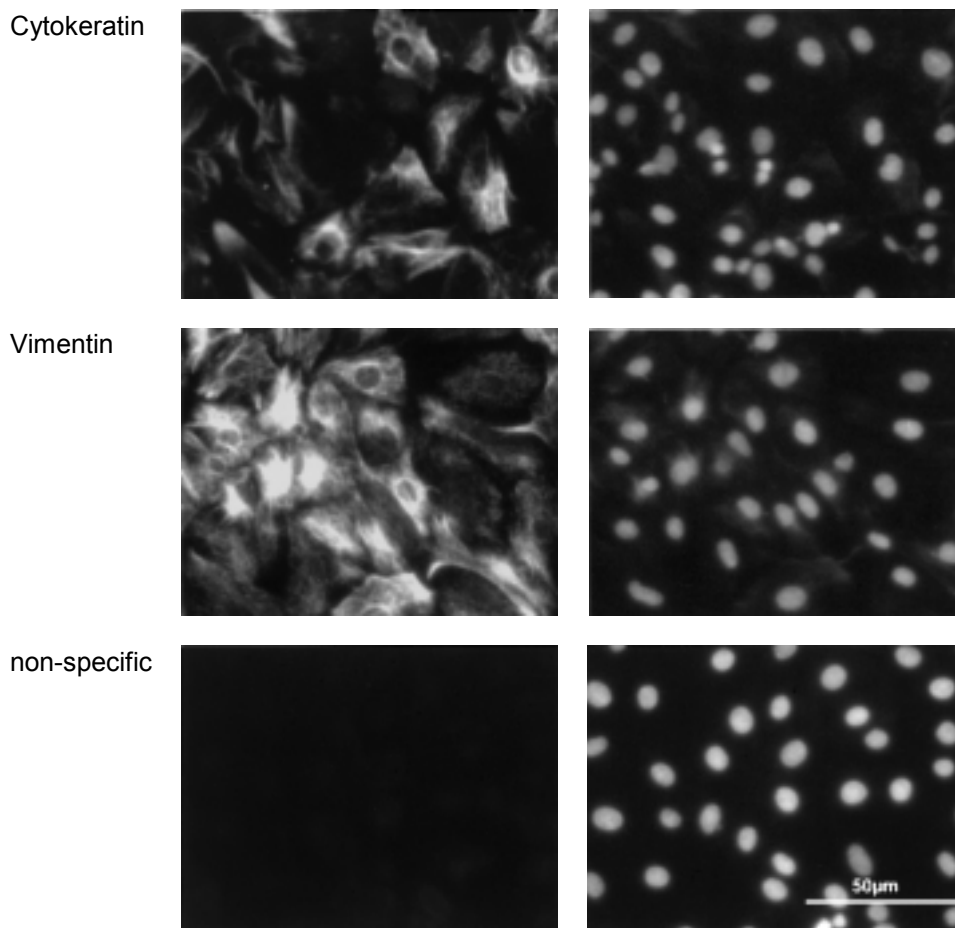


Fig. 1. Immunocytochemical characterisation of bovine pituitary cells cultured on plastic slides for four days. Fluorescent staining, left panels: cytokeratin (epithelial cells), vimentin (stromal cells) and non-specific within the cells. Hoechst stained nuclei on the same field, right panels. Scale bar represents 50 µm

Experiment 1

LH was secreted from pituitary monolayer cultures in a time- and dose-dependent manner in response to exogenous releasing hormone. Preliminary experiments showed that after a 6-h exposure to GnRH pituitary cells secreted more LH compared to that after 4 h, therefore we chose for the following experiments a 6-h incubation time with GnRH. At concentrations of 10^{-10} to 10^{-8} M GnRH, LH release was in the same range as the untreated control (data not shown), but a significant increase in LH release was observed at 10^{-6} or 10^{-5} M GnRH in comparison to the non-exposed control group (Fig. 2). After a 96-h culture period the pi-

tuitary cells in the non-stimulated group were also adherent to the culture plate and had a similar appearance to that of the cells in the GnRH-treated groups. Changes in the intensity of LH staining within the cells in dependence of different GnRH concentrations were not observed. Figure 3 illustrates representative photomicrographs of LH staining in pituitary cells. Only some cells reacted with the LH antiserum and showed an intensive immunostaining in the cytoplasm. There was no formation of positive immuno-products when the primary antibody was preabsorbed with homologous antigen, omitted or substituted by rabbit IgG.

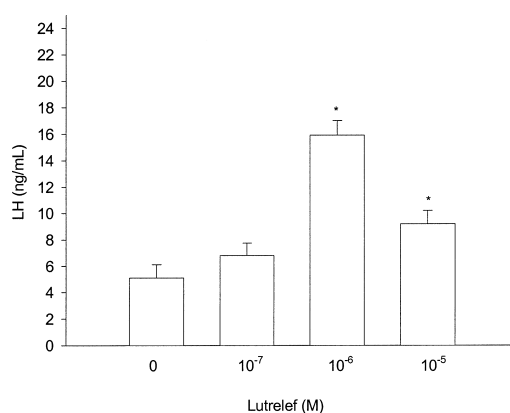


Fig. 2. Effect of GnRH on LH release from pituitary cells in culture after a 6-h incubation time. Results are presented as mean \pm SEM of data from 4 heifers. An asterisk designates significant difference between the control and experimental groups (* $P < 0.05$)

To determine whether the response of the pituitary cells to GnRH was elicited through the GnRH receptor, a competitive antagonist, Antarelix, was used to block the response to GnRH. Coadministration of Antarelix (10^{-4} M) significantly ($P < 0.05$) inhibited the GnRH-stimulated LH release by cells (Fig. 4a), whereas Antarelix alone had no effect on LH release of the cells (Fig. 4b). The staining of intracellular LH was present after the treatment of Antarelix compared to GnRH (data not shown).

Experiment 2

The indirect immunoperoxidase technique with anti-LH polyclonal antibody to localise LH immunoreactivity was performed on tissue sections of bovine pituitary glands. LH immunoreactivity was localised in some adenohypophyseal cells. When tissue sections reacted with anti-LH, only the cells which were round to oval in shape showed a strong positive staining in both Antarelix-treated (Fig. 5) and non-treated animals (data not shown). The immunostaining was completely inhibited if the LH antibody was preincubated with saturating concentrations of the homologous antigen.

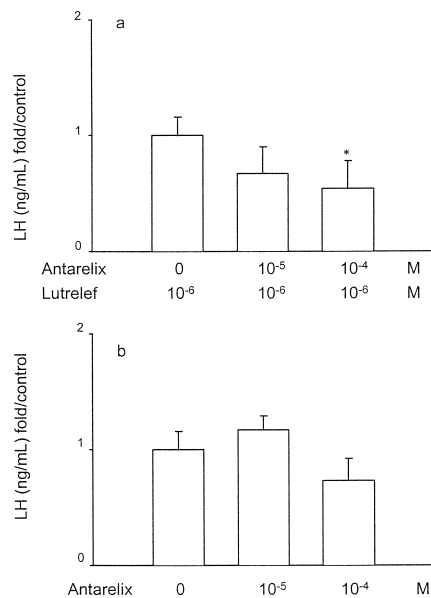


Fig. 4. (a) Effect of Antarelix on GnRH-stimulated LH secretion of pituitary cell cultures. An asterisk designates significant difference between the control and experimental groups (* $P < 0.05$). (b) Effect of Antarelix on basal LH secretion of pituitary cell cultures. Results are presented as mean \pm SEM of data from each 4 heifers

Monolayer cultures derived from the pituitaries after Antarelix treatment *in vivo* incubated with GnRH at concentrations of 10^{-7} to 10^{-5} M caused a significant ($P < 0.05$) increase in LH levels compared to the non-exposed control culture (Fig. 6). In monolayers derived from the pituitary in these animals, the staining of immunoreactive LH was not altered by *in vitro* GnRH treatment.

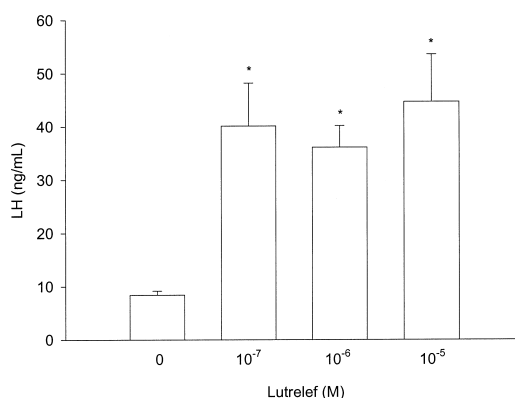


Fig. 6. Influence of Antarelix treatment administered *in vivo* on GnRH-stimulated LH secretion of pituitary cell cultures. Results are presented as mean \pm SEM of data from 2 heifers. An asterisk designates significant difference between the control and experimental groups (* $P < 0.05$)

Discussion

The results obtained from this study demonstrate that Antarelix inhibited GnRH-stimulated LH secretion, without significantly affecting basal LH release from bovine anterior pituitary cells in culture. Although this *in vitro* experiment was performed with bovine pituitary cells, it seems likely that these results were similar to those observed in rats (Danforth et al., 1991; Krsmanovic et al., 2000) and primates (Leal et al., 1989). The potency of the GnRH agonist was decreased in the presence of Antarelix, but the basal LH release in the cells was maintained. Therefore, we suggest on the one hand that locally produced LH contributes to the basal rate of LH secretion. On the other hand, Antarelix caused a direct antagonism of GnRH-stimulated LH secretion, probably by displacement of GnRH from its pituitary receptors. We found the presence of immunochemically positive LH staining in pituitaries of Antarelix-treated bovines and this result supports the hypothesis that Antarelix did not reduce the synthesis of this gonadotropin, but it inhibited GnRH-mediated LH secretion.

Our data on intracellular immunostaining of LH in GnRH-stimulated cultures are quite interesting. In fact, even if it is taken into account that the biosynthesis of a hormone involves mechanisms of hormone degradation and new hormone production and is therefore a complex process, a greater content of LH in GnRH-treated cells leads us to assume that there is a positive influence of GnRH on LH synthesis. We suggest that the physiological meaning of the possible positive action of GnRH on LH synthesis could be that this releasing hormone prepares the pituitary for the onset of the gonadotropin surge. Our explanation for the fact that Antarelix did not change the basal LH secretion could be that a low level of GnRH receptor activation by endogenous GnRH of pituitary origin (Krsmanovic et al., 2000) contributes to the basal rate of LH release *in vitro*. This fact is confirmed by results of previous experiments with antagonists of different species (Schally and Comaru-Schally, 1997). The occupation of pituitary GnRH receptors by antagonists at low doses has been reported to be reversible, explaining also the escape phenomenon, e.g. an increase of gonadotropin levels after some days with sustained antagonist application (Behre et al., 1997). In our experiments we were not able to detect pituitary GnRH receptors because the tissue amount from a single gland was too small for radio-receptor analyses carried out parallel with LH analyses.

Our result obtained in the mixed *in vivo/in vitro* experiment was a stimulation of LH release from pituitary cells by GnRH despite the previous antagonist treatment *in vivo*. The Antarelix dose used was effective to inhibit both pulsatile LH release and LH surge in a series of accompanying *in vivo* experiments (Schneider et al., unpublished). We assumed that the reduced gonadotropin plasma level decreased the follicular oestrogen production. Failure of the negative feedback by oestrogens stimulates hypothalamic GnRH release. Expression

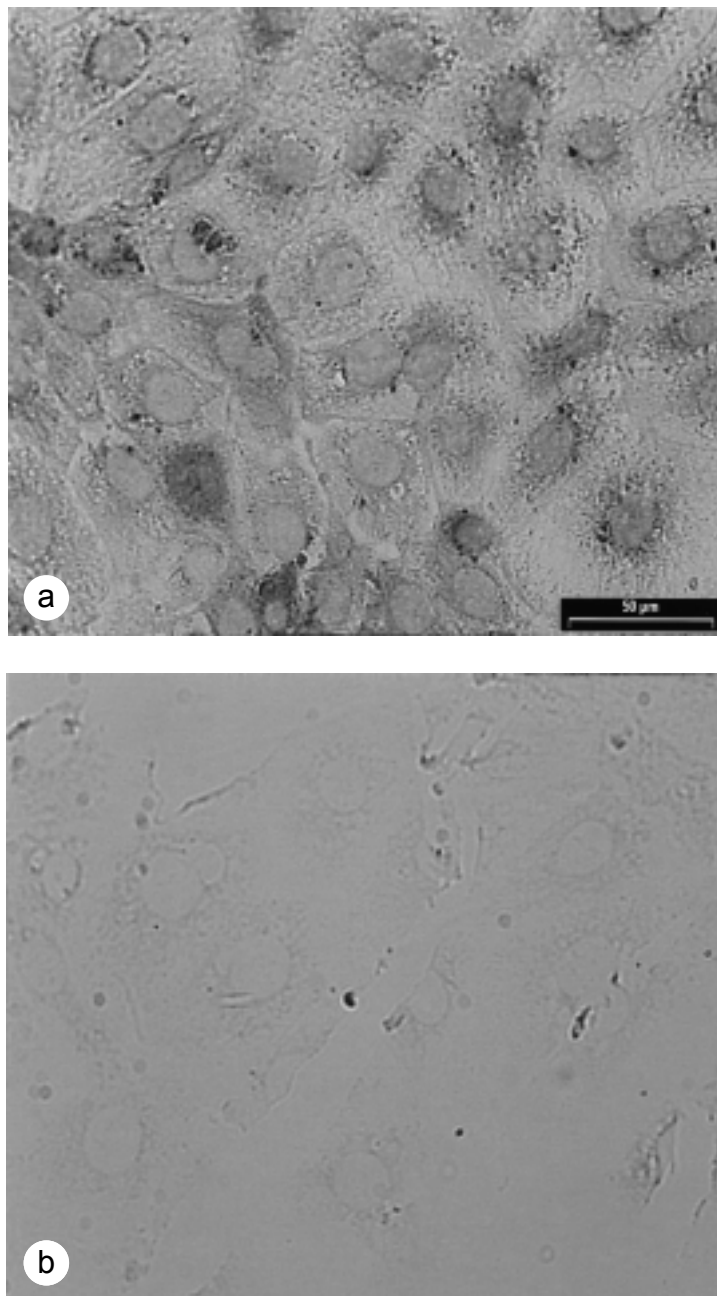


Fig. 3. Bovine anterior pituitary cell cultures were incubated with exogenous GnRH (10^{-6} M), fixed and stained with anti-LH. (a) LH staining is demonstrated immunocytochemically in these GnRH-treated flat cell sheets (brown staining). (b) Staining is abolished after preincubation with a saturating concentration of homologous antigen. Scale bar represents 50 µm

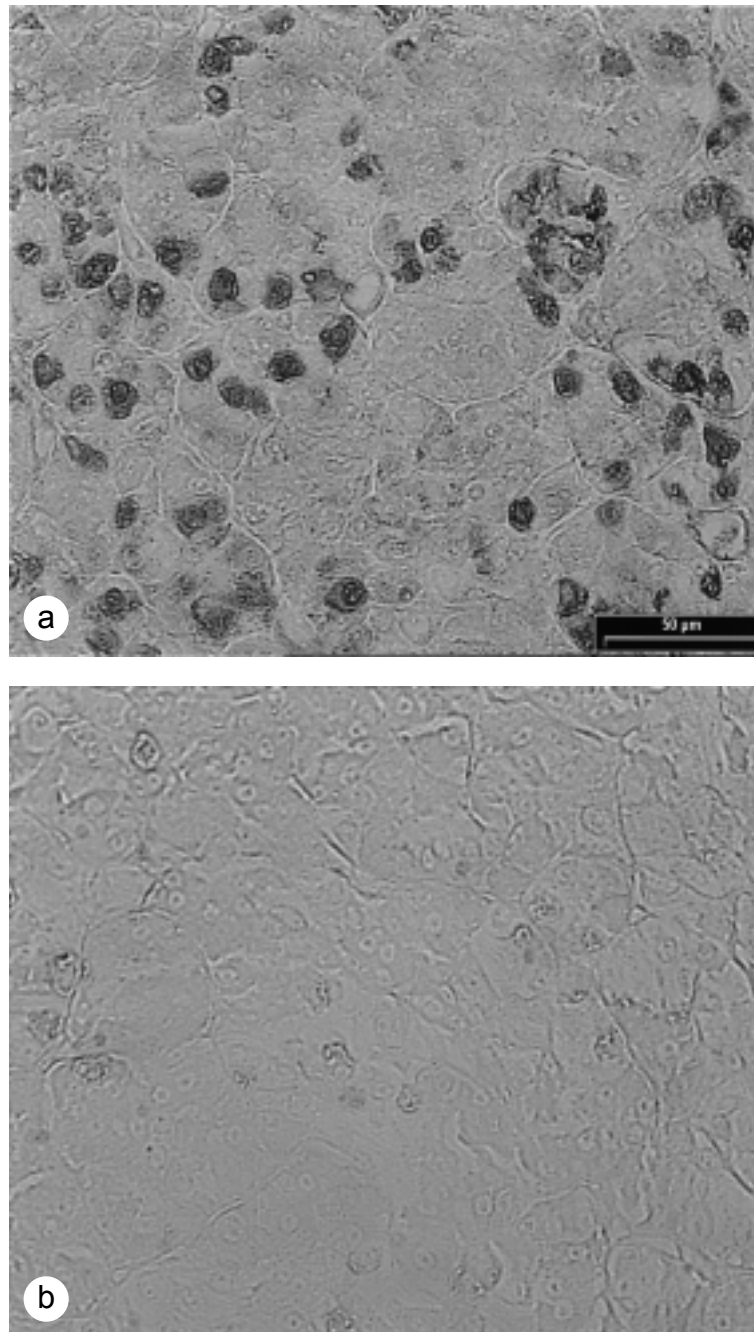


Fig. 5. Paraffin sections of bovine pituitary glands derived from Antarelix-treated heifers. (a) Some of the cells contain LH immunoreactivity (brown staining); (b) non-specific staining. Scale bar represents 50 μm

of pituitary GnRH receptor mRNA is maximal during the preovulatory period (Nett et al., 1987). The molecular events leading to maximal expression are probably triggered by decreased circulating concentrations of progesterone at luteolysis. Because GnRH is a positive homologous regulator of its own receptor (Turzillo and Nett, 1997; Adams et al., 1999), increased pulsatile GnRH concentration stimulates the expression of the GnRH receptor gene early in the preovulatory period. Oestradiol is also a positive regulator of GnRH receptor gene expression, and increased blood concentrations of oestradiol from developing follicles probably maintain high abundance of GnRH receptor mRNA later in the preovulatory period (Brooks and McNeilly, 1996).

In conclusion, our *in vitro* study on static cultures of bovine pituitary cells indicated that GnRH actively stimulates LH release in these cells after a 6-h exposure in a dose-dependent manner, whereas the secretion of LH is diminished if cells are pretreated with a GnRH antagonist. The presence of immunoreactivity to LH was observed after preincubation with GnRH antagonist and it seems to indicate that Antarelix allows the LH synthesis. In summary, we have shown that Antarelix acts as a pure antagonist of GnRH in the bovine species. No acute toxic effect of Antarelix was apparent on pituitary cells in culture.

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