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Bovine ovarian follicular cysts: *in vitro* effects of lecirelin, a GnRH analogue

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Abstract

This study investigates the mechanisms of action by which a GnRH analogue may modulate the contractility of the bovine ovarian follicular wall. The in vitro evaluation of the spontaneous basal contractility of bovine preovulatory and cystic follicles was performed, followed by testing the effects of lecirelin, a GnRH analogue, on their basal contractility. Strips of tissue in isolated organ bath were employed.

In addition, to better investigate the mechanism of action of lecirelin, the study of the effects of cumulative doses of nifedipine (a calcium channel blocker), phentolamine (an α-adrenoceptor antagonist) and reserpine (an inhibitor of the vesicular up-take of catecholamines) alone and, at the highest doses employed, associated to lecirelin, was set up.

The results demonstrate that in basal conditions and after the addition of lecirelin, the strips from preovulatory follicles contract significantly more than strips from cysts.

Furthermore, among the patterns of contractility evoked by the three drugs employed, the one induced by nifedipine was the only one unaffected by the addition of lecirelin.

The data obtained provide the hypothesis that one of the main mechanisms of action of GnRH, could involve calcium channels.

Keywords: Cystic follicle; Preovulatory follicle; Cow; Lecirelin; Ovulation

1. Introduction

Ovarian follicular cysts (FC) are the most common reproductive dysfunction in high producing dairy cows and represent an important cause of reproductive failure and economic loss for dairy industry [1].

According to Silvia et al [2], FC are defined as follicles that achieve a diameter of at least 17 mm and that persist for more than 6 days without luteinizing.

Follicular cysts may undergo spontaneous regression (within the first 50 days post-partum), persistence or replacement by a new cyst (cyst turnover) on the same or on the opposite ovary [3]. The persisting cysts induce abnormal oestrous patterns, such as irregular oestrous cycles, constant oestrus (nymphomania) or anoestrus.

Endocrine (mostly GnRH and LH) [2,4] as well as receptor deficiencies [5] together with vascular dysfunctions [6] are the main causes involved in FC development.

Beside the above mentioned factors, an alteration in the ovarian follicular contractility is implied in the determinism of FC [7].

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The process of ovulation relies on many events, among which, the contraction of the wall of the preovulatory follicle that increases pressure locally and forces the stigma to protrude from the surface of the ovary [8].

The theca externa layer of the follicular wall is, in fact, endowed with smooth muscle fibers, that are innervated by sympathetic nerves which, under stimulation by α-agonists, induce contraction [9,10].

Ferruz et al [11] hypothesized that also gonadotropins, beside modulating the central nervous activity, may act at the sympathetic nerve terminals, to locally regulate the release of Norepinephrine (NE).

However, the role of the ovarian adrenergic fibers in inducing the contractility of the follicular wall is still a matter of debate, in literature [12–14]. Given the above cited data, an alteration of the contractile apparatus of the ovary may often result in the development of FC [15].

Among the different therapies adopted for managing FC, the administration of GnRH analogues was shown to be efficient [1,16,17].

Basing on the well documented presence of GnRH receptors on the membrane of granulosa and theca cells of the follicular wall [18,19] and of smooth muscle fibers on the ovarian external theca, aim of this study was to investigate the mechanisms of action by which GnRH may modulate ovarian follicular contractility.

Thus the *in vitro* evaluation of the basal contractility of preovulatory follicles (PF) and FC, as well as the effects of a GnRH analogue, lecirelin, on the spontaneous contractility of strips isolated from bovine PF and FC were performed.

Lecirelin is a synthetic GnRH analogue which showed a great efficacy in the treatment of bovine ovarian follicular cysts, in previous in vivo experimentations [16,17].

In addition, the effects of a calcium channel blocker, nifedipine, of an α-adrenoceptor antagonist, phentolamine, and of an inhibitor of the vesicular up-take of catecholamines, reserpine, alone and associated to lecirelin were tested, to better investigate the mechanisms of action of lecirelin on PF- and FC-derived strips.

2. Materials and methods

The study was performed in 3 large farms of the South of Italy, in one year time (2009). 187 dairy cows destined to slaughter were enrolled in this experimentation. Still on the farms, two clinical examinations (including rectal palpation) and ultrasonographic transrectal evaluations of the ovaries were performed, 10 days apart, on all the cows. On the second gynaecological exam, the persistence of the same ovarian fluid-filled structures (wall thinner than 3 mm; absence of the *corpus luteum*) occurred in 126 out of 187 cows, which let us diagnose follicular cysts [2]. The remnant 61 cows were regularly cyclic and in oestrus at the moment of slaughtering.

All the cows underwent a blood collection from the coccigeal vein, for the quantitative dosage of serum progesterone, in order to help differentiate follicular from luteal cysts and to define the phase of estrous cycle.

After stunning, 187 pairs of ovaries were obtained. Moreover, the genital tracts and the functional ovarian structures of each cow were examined in order to confirm oestrus (preovulatory follicles) or to detect the follicular cysts, as described by Re et al [20]. The follicular and cystic features were *ex vivo* confirmed following the criteria of Silvia et al [2], thus the ovarian structures were classified as PF: diameter ranging between 17 and 20 mm; small cystic follicles (FC-S): diameter ranging between 17 and 22 mm; large cystic follicles (FC-L): diameter ranging between 23 and 30 mm.

The time course between slaughter and ovarian collection was 20 $\pm$ 5 minutes.

Soon after collection, a single physiological or pathologic ovarian structure was excised from each ovary; 2 full-thickness strips (5–10 mm) were cut from the wall of each follicle or cyst, following the method indicated by Walles et al [21]. Briefly, a strip was cut from the portion of the follicle or cyst protruding from the ovaries maintained in Kreb's solution (pH 7.4, composition: 113mM NaCl, 4.8 mM KCl, 2.2 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 5.5 mM glucose, 5.5 mM sodium ascorbate).

The strips were divided into 3 groups:

- 122 strips from PF;
- 128 strips from FC-S;
- 124 strips from FC-L.

The tissues were immediately placed in a flask containing chilled (+4 °C) modified pre-carboxygenated Krebs' solution and transferred to the laboratory. Mean transportation time was 15 $\pm$ 3 minutes.

The strips were placed in two jacketed organ baths (model 4050 Ugo Basile, Varese, Italy), containing 10 ml of Krebs' solution, suspended between parallel hooks and connected to an isometric force transducer (model 7003, model 7004, Ugo Basile, Italy). Thereaf-

ter, the strips were allowed to equilibrate under 1 gram tension, for 60 minutes.

The bath was maintained at +37 °C and continuosly bubbled with a mixture of 95% O_2 and 5% CO_2. During the equilibration period, the spontaneous contractility was recorded with a digital recorder (model 17400, Ugo Basile, Italy), and elaborated by a software (Data Capsule cat 17.400, Ugo Basile, Italy) for the evaluation of contractility.

Mean basal values of tension (grams) and frequency (number of peaks/time unit) of spontaneous contractions were evaluated for a 20 minute period followed by a wash out. The strips that did not exhibit contractility were discarded and a total number of 330 strips (110 from PF, 110 from FC-S and 110 from FC-L) were considered for the study, in order to uniform the groups.

The first step of the study concerned the evaluation of spontaneous contractility of strips derived from PF, FC-S and FC-L (10 for each group). These strips were let run for 3 1/2 hour [22]. The results obtained were used as trend of reference (control) for the following experiments. The second step consisted of testing the effect of lecirelin (Dalmarelin®, Fatro, Italy) on 51 strips cut from PF, FC-S and FC-L (17 for each group). The strips were exposed to cumulative concentrations (2.5 ng/mL–12.5 ng/mL–25 ng/mL–125 ng/mL–250 ng/mL–1.25 μg/mL–2.5 μg/mL) of lecirelin, for 20 minute periods, and then underwent a wash out. Since the minimum effective dose of lecirelin was 2.5 μg/mL, only this one was used in the subsequent experiments.

Three strips for each group were exposed to the solvent of Dalmarelin® at the same concentration (2.5 μg/mL), for 20 minute periods, in order to exclude its eventual capability of affecting contraction.

In the third step of the study the evaluation of the effects of nifedipine, phentolamine, and reserpine on the contractility of the ovarian strips was performed.

51 strips (17 for each group) were exposed, for 20 minute periods, to cumulative doses of the L-type voltage-activated calcium channel blocker, nifedipine (Sigma-Aldrich, Milano, Italy) dissolved in ethanol (Sigma-Aldrich, Milano, Italy), at the concentrations tested on the human myometrium [23]. The concentrations used were 10^{-5} M, 2×10^{-5} M, 5×10^{-5} M. Lecirelin (2.5 μg/mL) was added to the maximal effective concentration of nifedipine (5×10^{-5} M) and the evaluation of its effect lasted 20 minutes.

Three strips, for each group, were exposed to ethanol, at the same concentrations of nifedipine employed.

Further 60 strips (20 for each group) were exposed, for 20 minute periods, to cumulative doses of phentolamine hydrochloride (Sigma-Aldrich, Milano, Italy), an α-adrenoceptor antagonist, dissolved in ethanol, at concentrations of 50, 100, 150, and 200 μg/mL [24]. The maximal effective concentration (200 μg/mL) was followed, after 20 minutes, by the addition of 2.5 μg/mL of lecirelin, in the organ bath, for further 20 minutes.

Ulterior 51 strips (17 for each group) were exposed to cumulative doses of reserpine (Sigma-Aldrich, Milano, Italy), an inhibitor of the vesicular uptake of catecholamines, dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Milano, Italy), for 20 minute periods. The concentrations used were 5, 10, and 20 μg/mL, according to the doses tested in the uterus of guinea-pig [25]. Lecirelin (2.5 μg/mL) was added to the maximal effective concentration (20 μg/mL) of reserpine and the evaluation of its effects lasted 20 minutes.

Nine strips (3 for each group) were exposed to DMSO at the same concentrations of reserpine used.

More 60 strips were incubated overnight, at +4 °C, with Krebs' solution added with 5 μg/mL of reserpine, dissolved in DMSO. Incubation was performed to obtain an in vitro reserpinization, as indicated by previous studies [25,26]. The reserpinized strips were exposed to cumulative doses of reserpine, at concentrations of 5, 10, and 20 μg/mL, for 20 minute periods. The maximal effective concentration (20 μg/mL) was followed, after 20 minutes, by the addition of 2.5 μg/mL of lecirelin in the organ bath, for a further 20 minutes.

2.1. Statistical analysis

The mean values of tension (grams) and frequency (number of peaks/20 minutes) were evaluated in all the experiments, both before (basal) and after the addition of the substances. The values were expressed as Mean ± SEM and were evaluated for statistical significance ($P < 0.05$) with one way ANOVA and Tukey-Kramer's Multiple Comparison Test.

In the second step, the percentages of variation between the tension and frequency induced by lecirelin and the basal tone were tested. In the third step, the same variations were investigated between the values obtained at the maximal doses of each of the drugs employed and those detected after the addition of lecirelin.

3. Results

PF showed a spontaneous contractility characterized by amplitude and frequency significantly higher than those of FC-S and of FC-L. Moreover the values of tension were significantly higher in FC-S than in FC-L

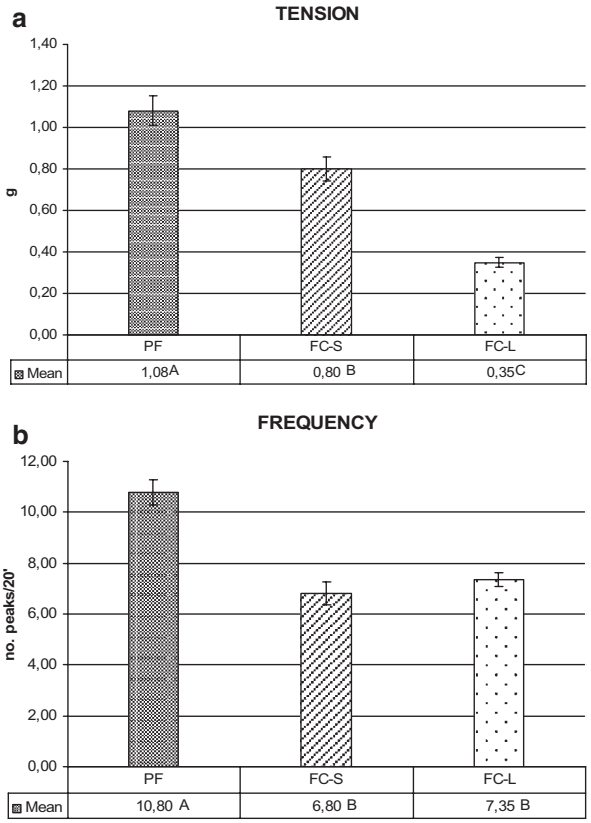


Fig. 1. (a) Mean values of tension in strips of preovulatory follicles (PF), small follicular cysts (FC-S) and large follicular cysts (FC-L). A,B: $P < 0.01$; A,C; B,C: $P < 0.001$. (b) Mean values of frequency of contractions in strips of preovulatory follicles (PF), small follicular cysts (FC-S) and large follicular cysts (FC-L). A,B: $P < 0.001$.

(Fig 1a and 1b). The solvent of Dalmarelin® had no pharmacological effects on the ovaries, at all the concentrations tested in these experiments.

Lecirelin (2.5 μg/mL) significantly increased the tension and frequency of contractions (Figs. 2a and 2b) in PF, FC-S, and FC-L in comparison with the basal values ($P < 0.001$). Statistically significant differences in tension and frequency of contractions were found between PF vs. FC-S and PF vs. FC-L.

The percentage of increase in tension was higher in FC-L (122%) than in FC-S (74%) and PF (36%), whereas the increase in frequency was higher in FC-S (69%), than in FC-L (61%) and in PF (29%) (Tables 1a and 1b). Ethanol, used to dissolve nifedipine, had no pharmacological actions on the strips at all the concentrations employed.

In PF, nifedipine induced a dose-dependent decrease both in tension and frequency of contractions, at a minimum effective dose of 2×10^{-5} M (Figs. 3a and 3b).

Furthermore, the addition of lecirelin (2.5 μg/mL) induced an increase in tension and in frequency of contractions of 3.5% and 4.9%, respectively (Tables 1a and 1b).

As to FC-S, the tension and frequency of contractions underwent a statistically significant decrease ($P < 0.01$) only when the maximum concentration of nifedipine (5×10^{-5}M) was used.

In the same strips, lecirelin determined an increase in tension and frequency of 1.9% and of 2.1%, respectively (Tables 1a and 1b).

In FC-L, the minimum effective doses able to exert effects on tension and frequency of contractions were 2×10^{-5} M and 5×10^{-5}. The addition of lecirelin induced an increase in frequency of contractions of 3.8% (Tables 1a and 1b).

As to tension, statistically significant differences ($P < 0.001$) were found only at PF vs. FC-L and FC-S vs. FC-L.

Phentolamine induced a relaxation of the strips cut from PF at a minimum effective dose of 150 μg/mL. In

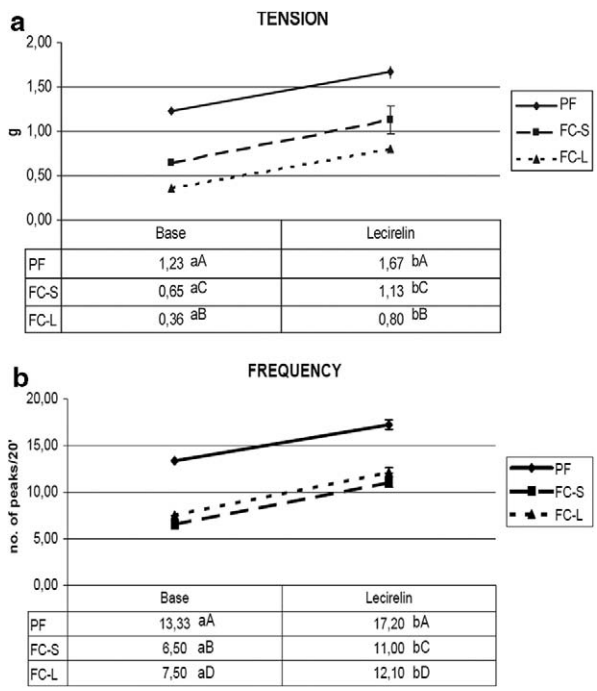


Fig. 2. (a) Mean values of tension in strips of preovulatory follicles (PF), small follicular cysts (FC-S) and large follicular cysts (FC-L), before (base) and after lecirelin administration. In the row: a,b: $P < 0.001$; in the column: A,C. $P < 0.05$; A,B: $P < 0.001$. (b) Mean values of frequency of contractions in strips of preovulatory follicles (PF), small follicular cysts (FC-S) and large follicular cysts (FC-L), before (base) and after lecirelin administration. In the row: a,b: $P < 0.001$; In the column: A,B: $P < 0.01$; A,C: $P < 0.05$; A,D: $P < 0.001$.

Table 1a
Percent increases of tension after the addition of lecirelin to: strips treated with lecirelin; strips treated with lecirelin at the maximum dose of nifedipine, phentolamine, reserpine and reserpine overnight. (PF: preovulatory follicles; FC-S: Small follicular cysts; FC-L: Large follicular cysts).

Tension	Lecirelin %	Lecirelin + nifedipine %	Lecirelin + phentolamine %	Lecirelin + reserpine %	Lecirelin + reserpine overnight %
PF	35	3,5	23	21	3,5
FC-S	74	1,9	12,1	18,8	5,2
FC-L	122	0	9	63,6	0

the same strips, lecirelin (2.5 μg/mL) increased tension and frequency of contractions of 23% and 10.9%, respectively (Tables 1a and 1b).

FC-S underwent significant decreases in tension (at the dose of 200 mcg/mL) and frequency (at the dose of 150 mcg/mL) of contractions. Increases in tension (12.1%) and frequency (5.6%) of contractions occurred in FC-S after the addition of lecirelin (2.5 μg/mL) (Tables 1a and 1b).

As to FC-L, the pattern of contraction was similar to that of FC-S. The increases in lecirelin-induced tension and frequency of contractions were 9% and 6.8, respectively (Tables 1a and 1b).

The statistical comparison of tension and frequency of PF vs. FC-S and PF vs FC-L revealed statistically significant differences at all the concentrations tested (Figs. 4a and 4b).

DMSO, used to dissolve reserpine, had no pharmacological actions on the strips, at all the concentrations employed.

In PF, reserpine significantly augmented the tension and frequency of contractions starting from a dose of 10 μg/mL. After the addition of lecirelin (2.5 μg/mL) the increases in tension and frequency were 21% and 23.4% (Tables 1a and 1b).

In FC-S, tension showed a significant increase at a minimum effective dose of 20 μg/mL of reserpine; increases of 18.8% in tension and 15.1% in frequency were observed, after the addition of lecirelin (Tables 1a and 1b).

As to FC-L statistically significant increases both in tension and frequency of contractions occurred after the addition of lecirelin. Lecirelin induced an increase in tension and frequency of 63.6% and 31.6% respectively, in comparison with the maximum dose of reserpine employed (20 μg/mL) (Tables 1a and 1b).

Tension showed statistically significant differences between PF and FC-L at all the concentrations tested. The maximum dose of reserpine employed (20 μg/mL) and the addition of lecirelin induced significant increases between FC-S and FC-L.

The comparisons of the frequencies of contractions between PF and FC-S and between PF and FC-L were significant at a dose of 10 μg/mL of reserpine and after the addition of lecirelin. No statistically significant differences were found between FC-S and FC-L (Figs. 5a and 5b).

The minimum effective dose of reserpine able to induce a significant decrease in tension of the reserpinized strips was 10 μg/mL for PF, FC-S and FC-L. The addition of lecirelin (2.5 μg/mL) increased tension of 3.5% in PF and 5.2% in FC-S (Tables 1a and 1b).

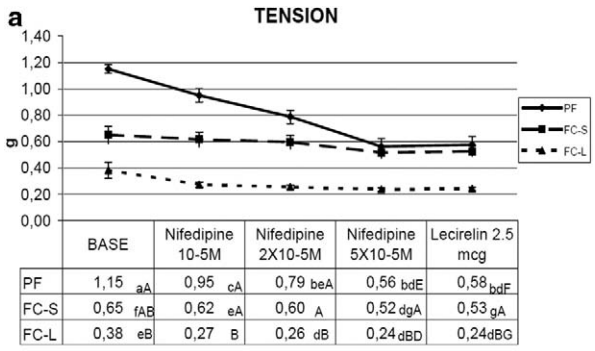
Frequency of contractions gave rise to different trends in the different tissues tested. Reserpine relaxed PF in a dose-dependent way ($P < 0.001$), whereas, the addition of lecirelin increased contractions of 43.9% (Tables 1a and 1b).

As to cysts, no significant responses were observed at all the concentrations of reserpine tested, whereas the addition of lecirelin increased frequency of contractions of 3.4% and 6.4%, in FC-S and FC-L, respectively (Tables 1a and 1b).

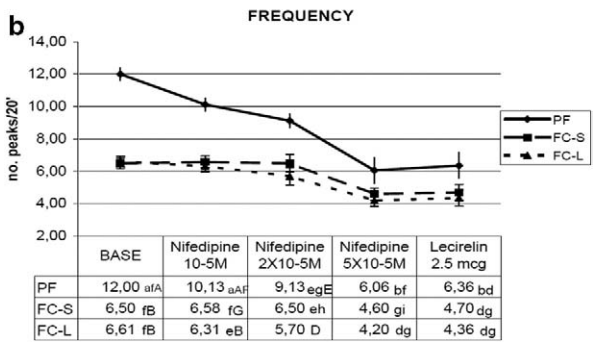
5 μg/mL of reserpine induced statistically significant differences in tension of contractions between PF

Table 1b
Percent increases of frequency after the addition of lecirelin to: strips treated with lecirelin; strips treated with lecirelin at the maximum dose of nifedipine, phentolamine, reserpine, and reserpine overnight.

Frequency	Lecirelin %	Lecirelin + nifedipine %	Lecirelin + phentolamine %	Lecirelin + reserpine %	Lecirelin + reserpine overnight %
PF	29	4,9	10,9	23,4	43,9
FC-S	69	2,1	5,6	15,1	3,4
FC-L	61	3,8	6,8	31,6	6,4



	BASE	Nifedipine 10-5M	Nifedipine 2X10-5M	Nifedipine 5X10-5M	Lecirelin 2.5 mcg
PF	1,15 aA	0,95 cA	0,79 beA	0,56 bdE	0,58 bdF
FC-S	0,65 fAB	0,62 eA	0,60 A	0,52 dgA	0,53 gA
FC-L	0,38 eB	0,27 B	0,26 dB	0,24 dBD	0,24 dBG



	BASE	Nifedipine 10-5M	Nifedipine 2X10-5M	Nifedipine 5X10-5M	Lecirelin 2.5 mcg
PF	12,00 afA	10,13 aAF	9,13 egE	6,06 bf	6,36 bd
FC-S	6,50 fB	6,58 fG	6,50 eh	4,60 gi	4,70 dg
FC-L	6,61 fB	6,31 eB	5,70 D	4,20 dg	4,36 dg

Fig. 3. (a) Mean values of tension in strips of preovulatory follicles (PF), small follicular cysts (FC-S) and large follicular cysts (FC-L). Dose-dependent effects of cumulative concentrations of nifedipine followed by a single dose of lecirelin. In the row: a,b; c,d: $P < 0.001$; e,d: $P < 0.05$; f,g: $P < 0.01$. In the column: A,B: $P < 0.001$; E,D: $P < 0.05$; F,G: $P < 0.01$. (b) Mean values of frequency in strips of preovulatory follicles (PF), small follicular cysts (FC-S) and large follicular cysts (FC-L). Dose-dependent effects of cumulative concentrations of nifedipine followed by a single dose of lecirelin. In the row: a,b: <0.001; e,d: $P < 0.05$; f,g; h,i: $P < 0.01$. In the column: A,B: $P < 0.001$; E,D: $P < 0.05$; F,G: $P < 0.01$.

and FC-S, PF and FC-L, FC-S and FC-L ($P < 0.001$), whereas a dose of 10 μg/mL induced significant differences in tension of contractions between PF and FC-S ($P < 0.01$), PF and FC-L ($P < 0.01$) (Figs. 6a and 6b).

4. Discussion

Contractility of *theca externa* layer of the wall of the preovulatory follicle is one of the main factors involved in its rupture, thus in ovulation. Both PgF2α and adrenergic innervations (mainly α-adrenergic receptors) are responsible for making this process occur [8,9].

An alteration of these mechanisms may lead to an inefficacious contraction of the smooth muscle fibres of the follicular wall and, consequently, to the development of the follicular cysts. The results obtained in the first step show a significant increase in the contractility of PF compared to FC and, among the latter, FC-S (diameter: 17–22 mm) contract more than FC-L (diameter: 23–30 mm).

The different patterns of contractility observed could have been determined by a likely reduction in contractile microfilaments in the cystic follicles. Estrogens, in fact, were shown to up-regulate the expression of these contractile elements, in the follicular wall [26]. In the FC, the reduced density of estrogen receptors [27] may have led to a minor expression of the smooth muscle fibers and, consequently, to a reduction in contractility.

Moreover, in FC-L, the over-stretching of the thecal contractile fibers may have further impaired their capability of contracting.

These experimental data demonstrate that lecirelin in vitro increased the spontaneous contractility of both PF and FC preparations.

These results indirectly confirm the presence of biologically active GnRH receptors in preovulatory follicles. Many Authors already showed the presence of these receptors in granulosa and luteal cells [18,28,29], whereas no data exist, to the best of our knowledge, about the presence of GnRH receptors, in follicular cysts. Calder et al [30] found an up-regulated expression of LH receptors in cysts, compared to dominant follicles.

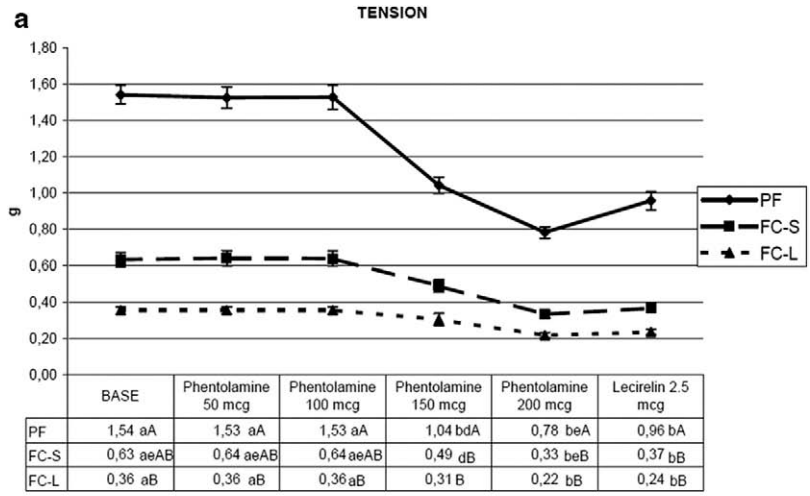
The results of this study evidence that lecirelin induced a higher increase in contractility in FC (particularly in FC-L), than in PF. This let us hypothesize the expression of GnRH receptors also in the cystic wall, or lecirelin is supposed to have bound to LH receptor, belonging both GnRH and LH receptors to the superfamily of membrane G protein-coupled receptors [8].

This last hypothesis could be strengthened by the results of Mitwally and Casper [31], showing that GnRH agonists may in vitro affect the expression and/or the activation of LH receptors in granulosa cells.

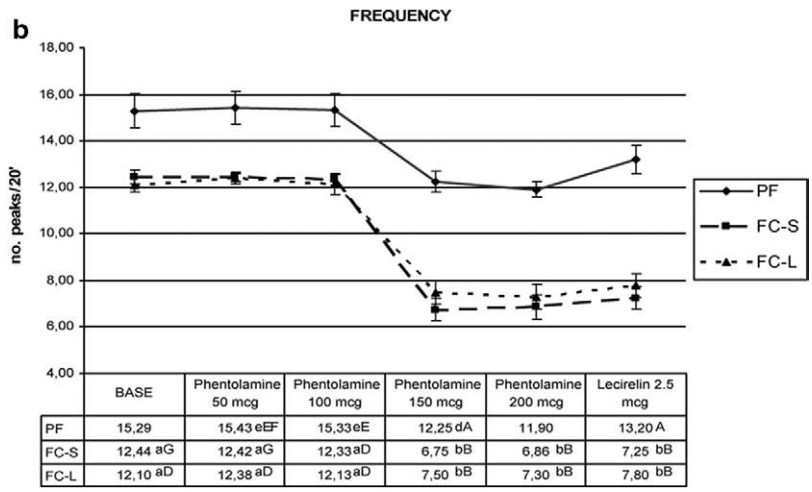
Beside the evaluation of the lecirelin-mediated effects on follicular cysts, the study aimed at elucidating the supposable mechanisms of action by which lecirelin may act.

Nifedipine, a calcium channel blocker, induced a relaxation of all the strips employed, at all the concentrations tested, in a dose-dependent way. Following the addition of lecirelin, the strips of both PF and FC didn't show any statistically significant difference in contractions.

The inability of the preparations to contract also after the addition of the GnRH analogue lets us hypothesize that the contractility evoked by lecirelin may rely on calcium flux. The activation of GnRH receptors by



	BASE	Phentolamine 50 mcg	Phentolamine 100 mcg	Phentolamine 150 mcg	Phentolamine 200 mcg	Lecirelin 2.5 mcg
PF	1,54 aA	1,53 aA	1,53 aA	1,04 bdA	0,78 beA	0,96 bA
FC-S	0,63 aeAB	0,64 aeAB	0,64 aeAB	0,49 dB	0,33 beB	0,37 bB
FC-L	0,36 aB	0,36 aB	0,36 aB	0,31 B	0,22 bB	0,24 bB



	BASE	Phentolamine 50 mcg	Phentolamine 100 mcg	Phentolamine 150 mcg	Phentolamine 200 mcg	Lecirelin 2.5 mcg
PF	15,29	15,43 eEF	15,33 eE	12,25 dA	11,90	13,20 A
FC-S	12,44 aG	12,42 aG	12,33 aD	6,75 bB	6,86 bB	7,25 bB
FC-L	12,10 aD	12,38 aD	12,13 aD	7,50 bB	7,30 bB	7,80 bB

Fig. 4. (a) Mean values of tension in strips of preovulatory follicles (PF), small follicular cysts (FC-S) and large follicular cysts (FC-L). Dose-dependent effects of cumulative concentrations of phentolamine followed by a single dose of lecirelin. In the row: a,b: $P < 0.001$; e,d: $P < 0.05$. In the column: A,B: $P < 0.001$. (b) Mean values of frequency of contractions in strips of preovulatory follicles (PF), small follicular cysts (FC-S) and large follicular cysts (FC-L). Dose-dependent effects of cumulative concentrations of phentolamine followed by a single dose of lecirelin. In the row: a,b: $P < 0.001$; e,d: $P < 0.05$. In the column: A,B: $P < 0.001$; E,D: $P < 0.05$; F,G: $P < 0.01$.

their agonists, in fact, fires a cascade mechanism involving the intracellular augment of Ca^{2+} [32–34].

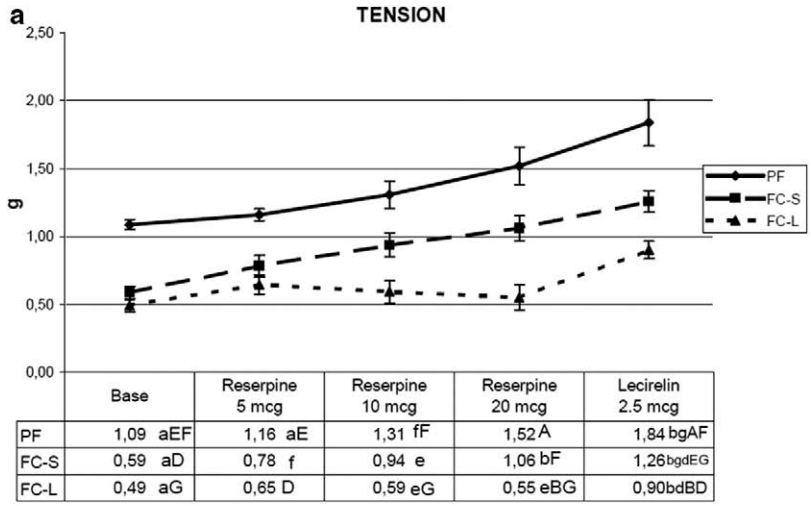
As to phentolamine, it induced a relaxation of the strips, more evident in the FC than in the PF. This result is in accordance with the results of Manni et al [35], who showed an augmented density of alpha adrenergic receptors in the follicular cysts, compared to preovulatory follicles. After the addition of lecirelin, an increase in the contractility occurred more evident in PF than in FC. This different pattern may result from the major phentolamine-dependent relaxation observed in the FC than in the PF.

However, the increase in contractions observed after the addition of lecirelin could suggest that an interaction with the postsynaptic adrenergic transmission is not among the main mechanisms of action of lecirelin.

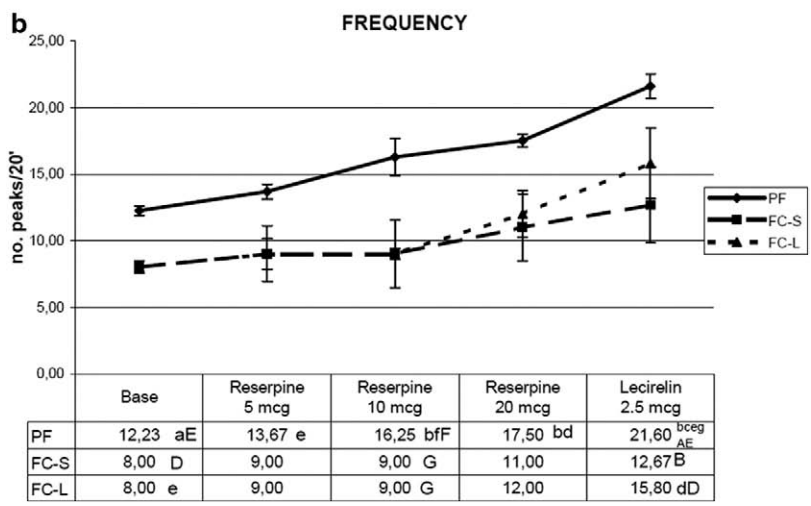
The administration of reserpine at medium-high doses (10, 20 μg/mL) transiently increased contractility of the strips. Lecirelin determined an increase of the reserpine-induced contractile activity, even if short-lasting, and not a relaxation, as it was expected.

The observed phenomenon could be due to the pharmacodynamic properties of reserpine, which inhibits the pre-synaptic vesicular uptake of NE [36], its intra-axonal accumulation and its consequent passive diffusion in the synaptic vallum.

In such conditions, the little amount of NE which is likely present in the synaptic vallum may reach the



	Base	Reserpine 5 mcg	Reserpine 10 mcg	Reserpine 20 mcg	Lecirelin 2.5 mcg
PF	1,09 aEF	1,16 aE	1,31 fF	1,52 A	1,84 bgAF
FC-S	0,59 aD	0,78 f	0,94 e	1,06 bF	1,26 bgdEG
FC-L	0,49 aG	0,65 D	0,59 eG	0,55 eBG	0,90 bdBD



	Base	Reserpine 5 mcg	Reserpine 10 mcg	Reserpine 20 mcg	Lecirelin 2.5 mcg
PF	12,23 aE	13,67 e	16,25 bfF	17,50 bd	21,60 $^{bceg}_{AE}$
FC-S	8,00 D	9,00	9,00 G	11,00	12,67 B
FC-L	8,00 e	9,00	9,00 G	12,00	15,80 dD

Fig. 5. (a) Mean values of tension in strips of preovulatory follicles (PF), small follicular cysts (FC-S) and large follicular cysts (FC-L). Dose-dependent effects of cumulative concentrations of reserpine followed by a single dose of lecirelin. In the row: a,b: $P < 0.001$; e,d: $P < 0.05$; f,g: $P < 0.01$. In the column: A,B: $P < 0.001$; E,D: $P < 0.05$; F,G: $P < 0.01$. (b) Mean values of frequency of contractions in strips of preovulatory follicles (PF), small follicular cysts (FC-S) and large follicular cysts (FC-L). Dose-dependent effects of cumulative concentrations of reserpine followed by a single dose of lecirelin. In the row: a,b; e,c: $P < 0.001$; e,d: $P < 0.05$; f,g: $P < 0.001$. In the column: A,B: $P < 0.001$; E,D: $P < 0.05$; F,G: $P < 0.01$.

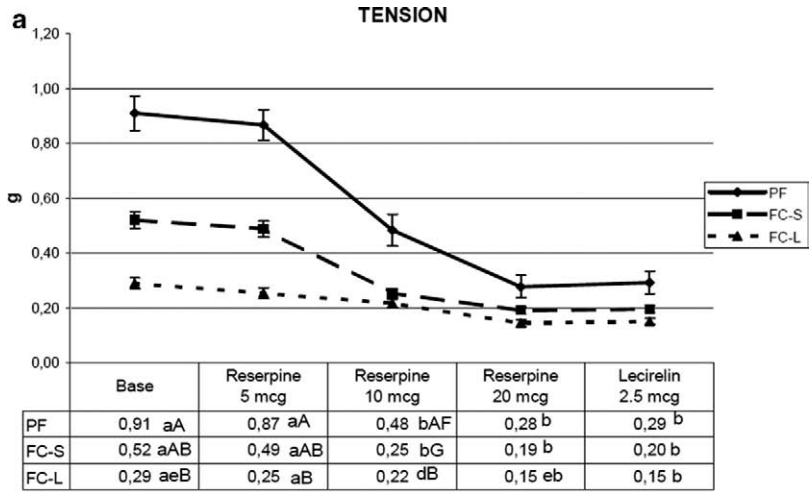
post-synaptic fiber, thus leading to the contraction observed. These results are in accordance with those obtained by Nayler [25] who evidenced a transient inotropic positive effect on heart preparates *in vitro* exposed to constant doses of reserpine.

The addition of lecirelin may have synergistically act with reserpine, prolonging its contractile effect.

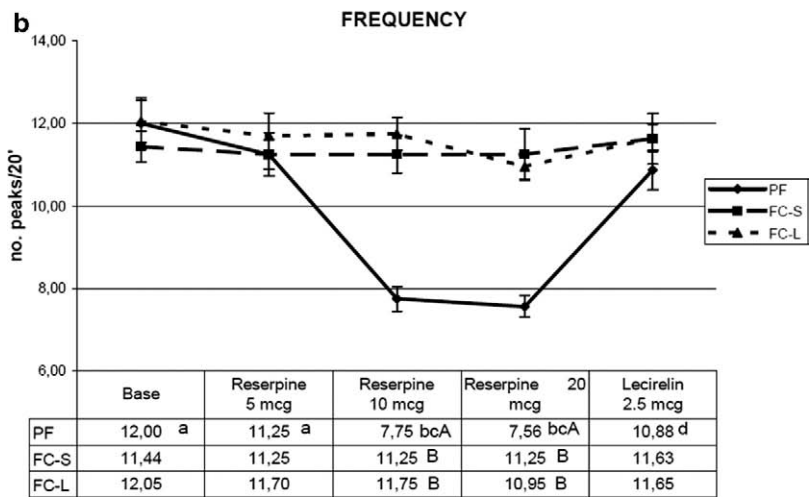
The overnight incubation of strips of follicular and cystic walls in Krebs' solution added with reserpine (5 μg/mL), may have induced a decrease in the uptake of NE in the pre-synaptic vesicles of the ovarian adrenergic fibers, thus leading to a gradual vesicular decrease, due to the enzymatic degradation exerted by Monoamino oxidase (MAO); these events may have blocked the adrenergic nervous transmission for a depletion of the neurotransmitter.

These results are in accordance with the negative inotropic effect observed by Nayler [25], in heart isolated strips, excised from *in vivo* reserpinized subjects. Furthermore, other *in vitro* studies demonstrated the depressant effects of reserpine on the myometrial contractile activity [24,37].

The absence of significant augments of contractility after the addition of lecirelin to the reserpinized strips



	Base	Reserpine 5 mcg	Reserpine 10 mcg	Reserpine 20 mcg	Lecirelin 2.5 mcg
PF	0,91 aA	0,87 aA	0,48 bAF	0,28 b	0,29 b
FC-S	0,52 aAB	0,49 aAB	0,25 bG	0,19 b	0,20 b
FC-L	0,29 aeB	0,25 aB	0,22 dB	0,15 eb	0,15 b



	Base	Reserpine 5 mcg	Reserpine 10 mcg	Reserpine 20 mcg	Lecirelin 2.5 mcg
PF	12,00 a	11,25 a	7,75 bcA	7,56 bcA	10,88 d
FC-S	11,44	11,25	11,25 B	11,25 B	11,63
FC-L	12,05	11,70	11,75 B	10,95 B	11,65

Fig. 6. (a) Mean values of tension in strips of preovulatory follicles (PF), small follicular cysts (FC-S) and large follicular cysts (FC-L). Dose-dependent effects of cumulative concentrations of reserpine overnight followed by a single dose of lecirelin. In the row: a,b: $P < 0.001$; e,d: $P < 0.05$. In the column: A,B: $P < 0.001$; F,G: $P < 0.01$. (b) Mean values of frequency of contractions in strips of preovulatory follicles (PF), small follicular cysts (FC-S) and large follicular cysts (FC-L). Dose-dependent effects of cumulative concentrations of reserpine overnight followed by a single dose of lecirelin. In the row: a,b; c,d: $P < 0.001$. In the column: A,B: $P < 0.001$.

suggests that, even the supposable intracellular increase of Ca^{2+} promoted by the GnRH analogue, may have not been able to promote contraction, in a condition of depletion of the neurotransmitter.

5. Conclusions

The results of this study let us hypothesize that lecirelin induces different contractility patterns in normal follicular preparations and in cystic samples and that these effects likely involve calcium channels. This conclusion derives from the observation that the lowest contractility observed after the addition of lecirelin occurred in strips previously treated with nifedipine, a calcium channel blocker. The binding of lecirelin to GnRH receptors, in fact, results in the augment of cytosolic Ca^{2+} [38,39], responsible for the initiation of the cascade of events leading to smooth muscle contraction.

These results suggest that GnRH, beside its central action, may modulate the contractility of the follicular wall, probably exerting a direct effect on calcium channels and indirectly affecting the nervous adrenergic transmission.

In conclusion, this study confirms the contractile properties of GnRH analogues on smooth muscle fi-

bres, spreading light on the deep mechanisms of action of these molecules and of the complex process of ovulation.

Acknowledgments

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