THE PHYSIOLOGICAL DILEMMA OF THE HIGH PROGESTERONE SYNDROME IN RABBIT DOES

Boiti C.[1]*, Guelfi G.[1], Brecchia G.[1], Gobbetti A.[2], Maranesi M.[1], Zerani M.[2]

[1]Dipartimento di Scienze biopatologiche ed Igiene delle produzioni animali e alimentari, Sezione di Fisiologia Veterinaria, Laboratorio di Biotecnologie fisiologiche, Università di Perugia, Via S. Costanzo 4, 06126 Perugia, Italy
[2]Dipartimento di Biologia Molecolare, Cellulare e Animale, Università di Camerino Via Gentile III da Varano, 62032 Camerino, Italy
*Corresponding author: cristiano.boiti@unipg.it

ABSTRACT

This work focused on the mechanisms that may cause multiple asynchronous ovulations and alter normal ovarian function in order to characterize the high progesterone (P+) syndrome in rabbit does, that, having abnormally high plasma progesterone concentration at the time of insemination, fail to become pregnant. At different luteal stages, at either days 4, 9, or 13 of pseudopregnancy, induced by GnRH injection (d-0), two groups of rabbits (n=5/group) were treated with saline or 0.8 µg GnRH. Blood samples were collected from d-0 to d-26 of pseudopregnancy. At d-4, GnRH injection prolonged ($P<0.05$) the functional CL life span by 3 to 4 d over that of controls. At d-9, GnRH caused a transient decline ($P<0.01$) of progesterone for the following 3 d but, thereafter, increased again and remained higher ($P<0.01$) than controls up to d-26. At d-13, progesterone fell to 1 ng/ml within one day following GnRH, but then gradually increased. Based of these progesterone profiles, it can be argued that, at both mid- and late-luteal phase, GnRH triggered luteolysis and induced ovulation followed by the formation of a new generation of CL. For the *in vitro* study, CL, collected at days 4, 9, and 13 of pseudopregnancy, were incubated with GnRH, GnRH-antagonist, PLA_2 inhibitor, and PLC inhibitor. GnRH decreased ($P<0.01$) progesterone secretion by d-9 and d-13 CL cultured *in vitro*; by converse, GnRH antagonist increased ($P<0.01$) progesterone release from d-4 CL. Co-incubation of GnRH with GnRH antagonist increased ($P<0.01$) progesterone release in d-4 CL, but had an opposite effect ($P<0.01$) on d-9 and d-13 CL. PLC inhibitor reversed the GnRH effects in both d-9 and d-13 CL, while PLA_2 inhibitor did not change progesterone release. These data suggest that rabbit CL express a functional receptor for GnRH, likely of type II, that utilizes the PLC post transductional cascade. Luteal FSH-R and LH-R mRNA relative abundances did not differ between d-4 and d-9 CL, but were two- to three-fold ($P\leq0.01$) higher, respectively, at d-13. StAR mRNA was highly expressed at d-4 of pseudopregnancy, but then markedly declined ($P\leq0.01$) at d-9 and d-13. Taken together, our results show that GnRH triggers i) functional regression when CL acquire luteolytic capacity from d 9 of pseudopregnancy onward, and ii) multiple asynchronous ovulations, thus partly explaining the P+ syndrome associated with the simultaneous coexistence of two population of "fresh" and "old" CL, although not yet the underlying causes.

Key words: GnRH, LH-R, FSH-R, StAR, Multiple ovulation.

INTRODUCTION

In rabbits, ovulation is a neuroendocrine reflex that is induced by mating or by exogenous administration of either gonadotropin releasing hormone (GnRH) or human chorionic gonadotropin. In the ovary of unmated or un-stimulated rabbits, therefore, functional corpora lutea (CL) should not be present and, in circulating plasma, progesterone concentration should remain at basal level. In a previous study, however, we reported abnormally high plasma progesterone (P+) concentrations at the time of insemination in does that failed to become pregnant (Boiti *et al*., 1996). Similar findings were later described by Theau-Clément *et al*. (2000), who observed the presence of two generations of functional CL, and by Rommers *et al*. (2006) in multiple caged does. More recently, this negative

relation between P+ concentration and fertility, defined as the high progesterone syndrome (Boiti *et al*., 2006), was confirmed in a large study (Theau-Clément *et al*., 2005).

In principle, since progesterone is mainly synthesised by steroidogenic luteal cells in the ovary, the occurrence of luteal function in the post partum of these rabbits suggests either a partial luteinisation of pre-ovulatory follicles, or a persistency of pre-existing gravidic CL due to fault luteolysis, or a pseudopregnant condition due to (multiple) spontaneous ovulation(s). In the latter case, however, the mechanisms whereby does become spontaneous ovulators are still unclear, but are likely associated to factors interfering with the control of the gonadal axis involving the hypothalamic centres responsible for GnRH release (Pau *et al*., 1986). Thus, the main objective of this work focused on the mechanisms that may cause spontaneous multiple ovulations and alter normal ovarian function in order to better characterize the P+ syndrome in rabbit does. In this study, GnRH administrations was used as a tool to mimic hypothalamic stimulation for multiple asynchronous ovulations.

MATERIALS AND METHODS

For the experiment, sexually mature New Zealand White female rabbits, weighing 3.5-4 kg, were housed individually in an indoor facility under controlled conditions of light (14 h L/10 h D) and temperature (18°C). Each animal had free access to food and water. Pseudopregnancy was induced with 20 IU PMSG (Folligon, Intervet, Milan, Italy) followed 3 days later by 0.8 µg GnRH analogue (Receptal, Hoechst-Roussel Vet, Milan, Italy). The day of GnRH injection was designated day 0.

Experiment 1
At different luteal stages, at either days 4, 9, or 13 of pseudopregnancy, two groups of rabbits (n=5/group/luteal stage) were treated i.m. with saline (0.2 ml) or 0.8 µg GnRH analogue. From each rabbit, blood samples were collected by venopuncture of the marginal ear vein from d-0 to d-26 of pseudopregnancy and immediately centrifuged at 3000 g for 10 min. Plasma progesterone concentrations were determined in duplicate by RIA according to the procedure reported elsewhere (Boiti *et al*., 2005) to assess the functional status of CL.

Experiment 2
For the *in vitro* study and analysis of expressions of luteal genes of interest, CL were harvested from other does sacrificed at either days 4, 9, or 13 of pseudopregnancy and randomly distributed (one CL/well) into incubation wells as previously described (Boiti *et al*., 2005). Each set of wells was divided into 6 groups of 5 wells: (I) medium alone; (II) GnRH (100 nM); (III) GnRH antagonist (GnRH-a, antide, 100 nM); (IV) GnRH+GnRH-a; (V) GnRH+phospholipase A2 (PLA_2) inhibitor (PLA_2-i, 4-bromophenacyl bromide, 1 µM); (VI) GnRH+phospholipase C (PLC) inhibitor (PLC-i, compound 48/80, 2 µM). Total RNA was extracted from CL of three rabbits for each luteal stage as previously described (Boiti *et al*., 2005). Five µg of total RNA was reverse transcribed in 20 µl of iSCRIPT cDNA (Bio-Rad) using random hexamer according to instructions. The optimal real-time efficiency and CT values were performed in PCR reaction mix containing 12.5 µL of iQ SYBR Green SuperMix (Bio-Rad), 1 µL of 10 µM forward and reverse primers (Table 1), 0.5 µL of cDNA, and water to 25 µL.

Table 1: Primers used for gene quantification by real-time RT-PCR

Gene	Accession Number		bp	Primers
LH-R	S57793 Homo sapiens	Forward	118	CTGGAGAAGATGCACAATGG
		Reverse		CAATTAGCCTCTGAATGGACTC
FSH-R	AY429104.1 Homo sapiens	Forward	150	GAGGAATGCCATTGAACTGAGG
		Reverse		GGAAGGTTGGAGAACACATCTG
StAR	HSSTAR8 Homo sapiens	Forward	130	AGAGCATCATCAACCAGGTC
		Reverse		GGGCACAGTTGGGAACAG
18S rRNA	X03205.1 Homo sapiens	Forward	148	CGATCAGATACCGTCGTAGT
		Reverse		TTCCTTTAAGTTTCAGCTTTGC

PCR was performed on iCycler iQ (Bio-Rad) with an initial incubation at 95 °C for 1.5 min, followed by 40 cycles at 95°C for 15 s, 53°C for 30 s, during which fluorescence data were collected. The threshold cycle (Ct value) was automatically computed. PCR products were purified and sequenced. The 18S Ct, housekeeping gene, was determined to normalize samples variation of starting cDNA. The melting curve analysis after the PCR end cycle was used to determine the specificity of each primer set.

Statistical analysis

The ratios of each PCR product for target genes, normalized against the 18S co-amplified product, were analyzed by two way ANOVA and Newman-Keuls multi comparison post-test. Data relative to overall *in vivo* and *in vitro* treatment effects on progesterone were analysed by ANOVA for repeated measurements. Comparison between effects was performed by Student's t-test.

RESULTS AND DISCUSSION

At d-4 of pseudopregnancy, GnRH injection prolonged ($P<0.05$) the functional CL life span by 3 to 4 d over that of controls (Figure 1, A), but it remains to be established whether this longer luteal phase was indeed due to novel CL deriving from another ovulation or rather to luteinization of ovarian follicles. At d-9, GnRH administration caused a transient decline ($P<0.01$) of the peripheral plasma progesterone concentrations for the following three days but, thereafter, progesterone increased again and remained higher ($P<0.01$) than controls up to d-26 (Figure 1, A). At d-13, progesterone concentration fell to 1 ng/ml within one day following GnRH, but then gradually increased (Figure 1, A). Based on progesterone profiles, it can be argued that, at both mid- and late-luteal phase, GnRH triggered luteolysis of pre-existing CL and induced the ovulatory mechanism followed by the formation of a new generation of CL. Whether GnRH causes CL functional regression directly, via luteal GnRH-R, or indirectly as a consequence of the withdrawal of the luteotropic support given by estrogens remains to be further investigated. At day 11 of pseudopregnancy, GnRH injection induced luteinization of ovarian follicles, and functional regression of CL as assessed by serum progesterone levels decline within 3 days (Rippel *et al*., 1976). Depending on the dosage administered between day 8 and 13 of gestation, GnRH was found to either cause abortion by luteolysis or support foetal survival by stimulating luteotropic activity (Hilliard *et al*., 1976).

GnRH markedly reduced ($P<0.01$) progesterone secretion of both mid- and late-luteal stage CL cultured *in vitro*, but had no effect on d-4 CL (Figure 1, B). By converse, GnRH-a enhanced ($P<0.01$) progesterone release from d-4 CL, but had no effect on older CL (Figure 1, B). The co-incubation of GnRH with GnRH-a increased ($P<0.01$) progesterone release of d-4 CL, but decreased ($P<0.01$) that of d-9 and d-13 CL (Figure 1, B). The addition of PLA_2-i did not change the progesterone release into the medium (Figure 1, B). By converse, in both d-9 and d-13 CL, PLC-i reversed the inhibitory effects of GnRH as progesterone productions matched those of CL cultured in medium alone (Figure 1, B). Taken together, these data suggest that rabbit CL express a functional receptor for GnRH (GnRH-R), which likely belongs to the type II family in analogy to what found in other species (Hapgood *et al*., 2005), and that the cell signalling pathways activated by GnRH operates along the PLC cascade.

Luteal FSH-R and LH-R mRNA relative abundances did not differ between d-4 and d-9 CL, but were two to three-fold ($P\leq0.01$) higher, respectively, at d-13 (Figure 2). Steroidogenic acute regulatory protein (StAR) mRNA was highly expressed at d-4 of pseudopregnancy, but then markedly declined ($P\leq0.01$) at d-9 and d-13 (Figure 2). Thus, independently of luteal stage, rabbit CL expressed transcripts for both FSH and LH receptors. Although their steady state relative abundances were up-regulated at d-13 of pseudopregnancy, by approximately the same factor, their roles in ensuring a normal luteal function in the presence of pituitary gonadotropins, are still unclear. In rabbits, in fact, estradiol-17β is the primary luteotropic hormone and, beyond the first day following ovulation, the secretion of LH or FSH is not required for a normal course of development, maintenance and regression of CL (Bill and Keyes, 1983).

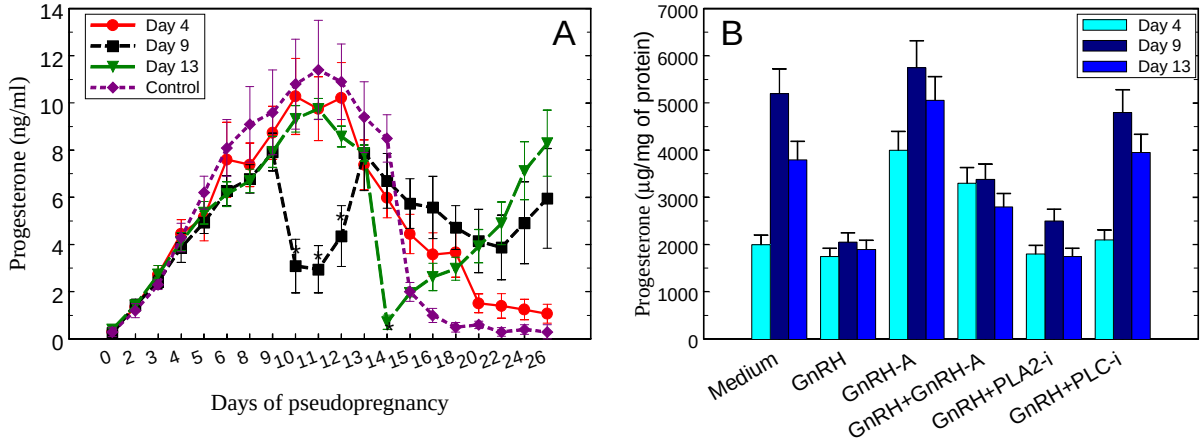

Figure 1: In panel A, plasma progesterone levels after GnRH injection to rabbits at days 4, 9, and 13 of pseudopregnancy. In panel B, progesterone secretion by CL cultured *in vitro* with both GnRH agonist and antagonist and inhibitors for PLA2 and PLC

FSH receptors has been detected in the bovine CL (Manns *et al*., 1984) without any obvious correlation between stage of the estrous cycle and physiological function as well as in CL of women (Minegishi *et al*., 1997). High number of LH receptors having high affinity binding sites throughout early pregnancy have been found in the primary CL of mares (Saint-Dizier *et al*., 2003) and pigs.

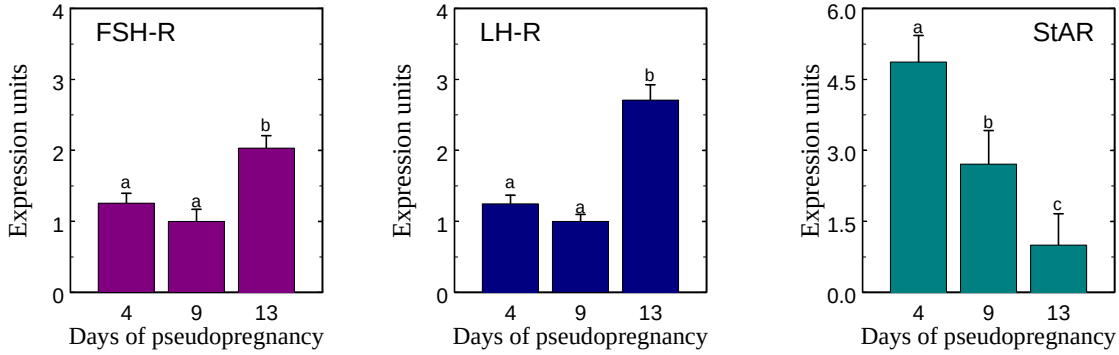

Figure 2: FSH-R, LH-R, and StAR relative mRNAs levels in rabbit CL at different stages of pseudopregnancy. Different letters above bars indicate a significantly different value (P<0.01) among treatments

StAR is an indispensable protein that regulates at the mitochondrial level the conversion of cholesterol into pregnenolone, the rate limiting step in steroid synthesis. The StAR expression in monkey CL varied in accordance with the changes of serum progesterone levels, being the highest at the developing stage (5 d after ovulation) and the lowest at later luteal phases, 18 d after ovulation (Liu *et al*., 2003) in good accordance with present results.

CONCLUSIONS

Taken together our results, derived from both *in vitro* and *in vivo* models, show that GnRH triggers i) either directly o indirectly, functional luteal regression when CL acquire luteolytic capacity from day 9 of pseudopregnancy onward, and ii) multiple asynchronous ovulations, thus partly explaining the P+ syndrome associated with the simultaneous coexistence of two population of “fresh” and “old” CL, although not yet the underlying causes.

ACKNOWLEDGEMENTS

Work supported by a grant from the Italian "MiUR".

REFERENCES

Bill C.H., Keyes P.L. 1983. 17 beta-estradiol maintains normal function of corpora lutea throughout pseudopregnancy in hypophysectomized rabbits. *Biol. Reprod., 28, 608-617.*

Boiti C., Guelfi G., Brecchia G., Dall'Aglio C., Ceccarelli P., Maranesi M., Mariottini C., Zampini D., Gobbetti A., Zerani M. 2005. Role of endothelin-1 system in the luteolytic process of pseudopregnant rabbits. *Endocrinology, 146, 1293-1300.*

Boiti C., Besenfelder U., Brecchia G., Theau-Clèment M., Zerani M. 2006. Reproductive physiopathology of the rabbit doe. *In: L. Maertens and P. Coudert (Eds.). Recent advances in rabbit Science, COST Action 848, 3-20.*

Boiti C., Canali C., Monaci M., Stradaioli G., Verini Supplizi A., Vacca C., Castellini C., Facchin E. 1996. Effect of postpartum progesterone levels on receptivity, ovarian response, embryo quality and development in rabbits. *In: Proc. 6th World Rabbit Congress, June 1996, Toulouse, France, Vol. 2, 45-49.*

Hapgood J.P., Sadie H, van Biljon W., Ronacher K. 2005. Regulation of expression of mammalian gonadotrophin-releasing hormone receptor genes. *J. Neuroendocr., 17, 619-638.*

Hilliard J., Pang C.N., Sawyer C.H. 1976. Effects of luteinizing hormone-releasing hormone on fetal survival in pregnant rabbits. *Fertil. Steril., 27, 421-425.*

Liu K., Feng Q., Gao H.J., Hu Z.Y., Zou R.J., Li Y.C., Liu Y.X. 2003. Expression and regulation of plasminogen activators, plasminogen activator inhibitor type-1, and steroidogenic acute regulatory protein in the rhesus monkey corpus luteum. *Endocrinology, 144, 3611-3617.*

Manns J.C., Niswender G.D., Braden T. 1984. FSH receptors in the bovine corpus luteum. *Theriogenology, 22, 321-328.*

Minegishi T., Tano M., Igarashi M., Rokukawa S., Abe Y., Ibuki Y., Miyamoto K. 1997. Expression of follicle stimulating hormone receptor in human ovary. *Eur. J. Clin. Invest., 27, 469-474.*

Pau K.Y., Orstead K.M., Hess D.L., Spies H.G. 1986 Feedback effects of ovarian steroids on the hypothalamic-hypophyseal axis in the rabbit. *Biol. Reprod., 35, 1009-1023.*

Rippel R.H., Jonhson E.S., Kimura E.T. 1976 Regression of corpora lutea in the rabbit after injection of a gonadotropin-releasing peptide. *Proc. Soc. Exp. Biol. Med., 152, 29-32.*

Rommers J.M., Boiti C., De Jong I.C., Brecchia G. 2006. Performance and behaviour of rabbit does in a group-housing system with natural mating or artificial insemination. *Reprod. Nutr. Dev., 46, 677-687.*

Saint-Dizier M., Chopineau M., Dupont J., Daels P.F., Combarnous Y. 2003. Expression and binding activity of luteinizing hormone/chorionic gonatropin receptors in the primary corpus luteum during early pregnancy in the mare. *Biol. Reprod., 69, 1743-1749.*

Theau-Clément M., Boiti C, Brecchia G. 2005. Characterisation of pseudopregnant rabbit does at the moment of artificial insemination. Preliminary results. *COST Meeting, 2005 June, Palermo, Italy, 32.*

Theau-Clément M., Boiti C., Mercier P., Falières J. 2000. Description of the ovarian status and fertilising ability of primiparous rabbit does at different lactation stage. *In: Proc. 7th World Rabbit Congress, 2000 July, Valencia, Spain, Vol. A, 259-266.*