ULTRASOUND EVALUATION OF OVARIAN FOLLICULAR DYNAMICS DURING EARLY PSEUDOPREGNANCY AS A TOOL TO INQUIRE INTO THE HIGH PROGESTERONE (P+) SYNDROME OF RABBIT DOES

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ABSTRACT

The investigation of the underlying causes for the high progesterone (P+) syndrome should presuppose further studies to more precisely characterise pseudopregnant rabbit does. The purpose of the present research was to examine follicular dynamics in the postovulatory rabbit ovary by means of real-time B-mode ultrasound scanning. A particular attention was focused upon the population of large-sized follicles present through the first 6 days after induced pseudopregnancy, since this follicular category, being steroidogenically active, may be crucial to the survival of the developing CL. Pseudopregnancy was induced in twenty-four hybrid rabbit does caged individually by injection of 100 IU human chorionic gonadotropin (hCG). Sequential monitoring of dynamic changes in the follicular population was performed by ultrasonography on day 0, 2 and 6 of pseudopregnancy (day 0 being the day of hCG injection). The follicle distribution in ovaries of untreated does (day 0) was marked by approximately equal number of small follicles (46.1%±6.7 <2.0 mm) and large follicles (54.4%±6.1 ≥2.0 mm). However, at 2 days after injection of an ovulatory dose of hCG, the large follicles population was markedly depleted with only 10.2%±0.9 of the follicles present classified as large. The ovulation rate averaged 10.8±0.7 per rabbit. By day 6 of pseudopregnancy the size distribution showed that a repopulation of the ovary with large follicles occurred with 71.3%±6.8 in this category. The large follicles lost at ovulation had apparently been replaced by the time of luteal estrogen dependence at day 6 of pseudopregnancy. The results presented in this report, demonstrating that the rabbit ovary is depleted of large follicles following ovulation but that large, steroidogenically active follicles are again present by day 6, could be considered as a preliminary step to understand what mechanisms protect the CL from luteolysis until day 6 of pseudopregnancy, when CL shift from complete refractoriness to partial and complete responsiveness to PGF2α treatment. Since little is known about what factors effectively trigger luteolysis in normal, physiological conditions as well as what mechanisms protect CL from luteolysis in the first days of pseudopregnancy, the innovative and non-invasive approach supplied by the real-time ultrasonography could represent an additional tool for investigation.

Key words: Rabbit, High progesterone syndrome, Pseudopregnancy, Ovarian follicles, Ultrasonography.

INTRODUCTION

The corpus luteum (CL) is a transient endocrine gland that secretes progesterone to support pregnancy. The CL are formed from ovulated follicles in a process that involves angiogenesis and tissue remodelling under the influence of several endothelial-derived factors acting locally in a paracrine/autocrine manner, together with different luteotropic hormones such as LH, estradiol 17-β and probably also PGE2 (Boiti, 2004). Functional CL should not be present in the ovary of unmated rabbits or in the *post partum* period being ovulation the result of a neuroendocrine reflex, peculiarly induced in this species by mating or exogenous GnRH administration when AI is performed. The

observation that luteal activity may exert a negative effect on the reproductive efficiency of rabbit does was reported in different studies (Boiti *et al.*, 1996; Theau-Clément *et al.*, 2000; Rommers *et al.*, 2006), featuring unexpected high plasma progesterone concentrations (P+) and CL in the *post partum* at the time of AI. This condition of pseudopregnancy (P>1 ng/ml) while did not impede GnRH-induced ovulation, was indeed responsible for anti-reproductive effects, given that most of these P+ does were not receptive and did not became pregnant (Rommers *et al.*, 2006). Thus, the high progesterone syndrome could signify an actual limitation on production, also because of its frequency: up to 32.5% in primiparous does (Theau-Clément *et al*., 2005). In the experiments cited above, however, only some hypothesis were advanced to justify the abnormal status of pseudopregnancy and the causes of ovulations occurring prior to insemination are currently unidentified (Theau-Clément, 2007).

The investigation of the underlying causes for the P+ syndrome should anyway presuppose further studies to more precisely characterise pseudopregnant does. The purpose of the present research was to examine follicular dynamics in the postovulatory rabbit ovary by means of real-time B-mode ultrasound scanning. A particular attention was drawn to the population of large-sized follicles present through the first 6 days after induced pseudopregnancy, since this follicular category, being steroidogenically active, may be crucial to the survival of the developing CL.

MATERIALS AND METHODS

Animals and experimental design

Twenty-four multiparous hybrid rabbit does were utilized to carry out the present experiment. The animals were kept isolated from one another, being caged individually, under controlled conditions of light (14L:10D schedule) and with food and water available *ad libitum*. Pseudopregnancy was induced by injection into a lateral ear vein of 100 IU human chorionic gonadotropin (hCG), which bypasses the hypothalamus-pituitary axis to directly target the ovarian follicles. Sequential monitoring of dynamic changes in the follicular population was performed on day 0, 2 and 6 of pseudopregnancy (day 0 being the day of hCG injection) by means of real time B-mode ultrasound scanning. The protocol involving the care and use of the animals for this experiment was approved by the Bioethic Committee of the University of Sassari.

Scanning procedure

Rabbits abdominal skin was shaved at the beginning of the experiment and before each examination the area was covered with scanning gel. The ultrasound exams were performed using a 7.5-mHz linear-array transducer (Model UST-5512U-7.5; Aloka Inc., Tokyo, Japan) with a real-time B-mode ultrasound scanner (SSD-500; Aloka Inc.). During each scanning session, the setting of the scanner that affect image attributes (e.g. overall time-gain, near-field and far-field gains, compensation and beam focus) were kept at predetermined levels. Images were displayed at the maximum magnification. One or more on-screen images in several planes of both ovaries were accurately studied to assess the follicular dynamics. In order to investigate the follicular population, individual follicles on each ovary were identified, measured and classified according to the following size criteria: small follicles (<2.0 mm) and large follicles (≥2.0 mm).

Statistical Analysis

Student's t test (option PDIFF) was used to analyse for statistical differences among follicular sizes according to 0, 2 and 6 days after hCG injection. Size distributions are presented as means and standard errors (% ± SE).

RESULTS AND DISCUSSION

The follicles monitored by means of ovarian ultrasound scanning appeared as well-defined anechoic circular areas. The total amount of detectable follicles in the experimental rabbits was 281 (n=24), 97 (n=21) and 411 (n=21) at 0, 2 and 6 days after hCG injection, respectively. The mean distribution of sizes of ovarian follicles ≥1.8 mm in diameter at 0, 2 and 6 days after hCG injection is presented in Figure 1. The follicle distribution in ovaries of untreated does (day 0) was marked by approximately equal number of small follicles (46.1% ± 6.7 <2.0 mm) and large follicles (54.4% ± 6.1 ≥2.0 mm). The small difference verified among animals was not unexpected since the rabbit doe maintains a relatively constant supply of preovulatory follicles. As a matter of fact in domestic rabbits, in which there is no spontaneous gonadotropin surge, waves of follicles undergo continuous maturation, such that at nearly any given time, ovulable follicles are present (Fleming *et al.*, 1984).

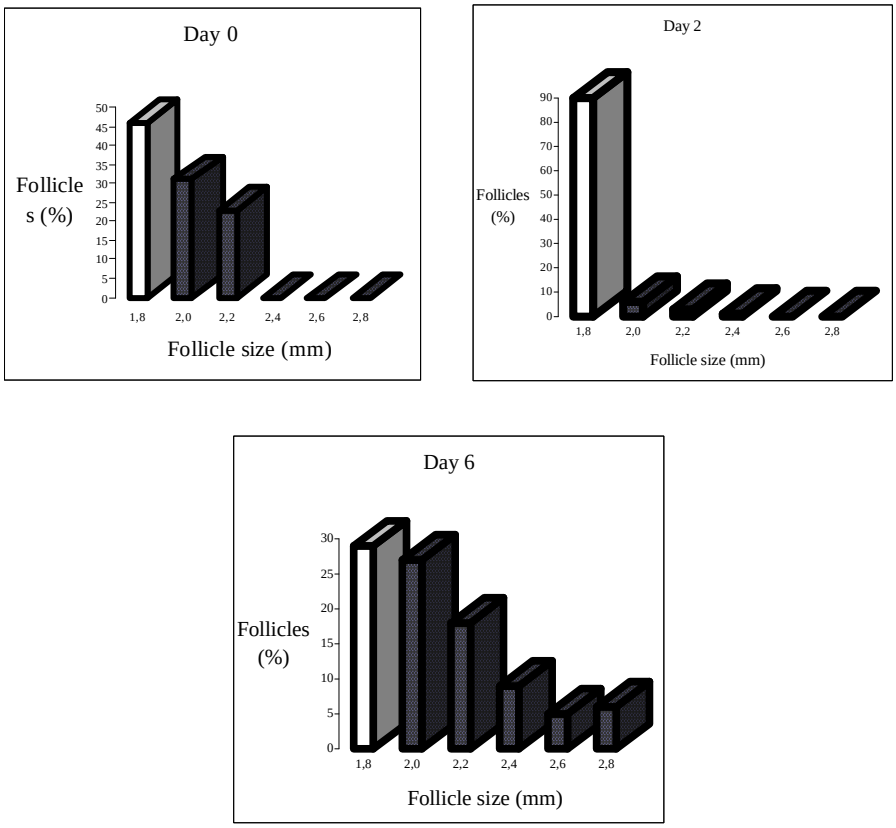

Figure 1: Size distribution of follicles monitored by ovarian ultrasound scanning at 0, 2 and 6 days after hCG injection. Only follicles ≥ 1.8 mm in diameter were identified, measured and classified

At 2 days after injection of an ovulatory dose of hCG, the large follicles population had been markedly depleted with only 10.2% ± 0.9 of the monitored follicles classified as large. Three rabbits failed to ovulate causing their removal from the data set. By day 6 of pseudopregnancy the size distribution showed that a repopulation of the ovary with large follicles had occurred with 71.3% ± 6.8 in this category. CL appeared by ultrasound scanning as hyperechoic structures. The ovulation rate (number of CL on both ovaries) averaged 10.8 ± 0.7 per rabbit (n=21) and was in agreement with Adams *et al.* (1961) who reported four to six CL developing in each ovary after administration of an ovulatory hCG dose.

Regarding the stereodogenic capability of the follicles present through the first days of pseudopregnancy, early studies (Hilliard and Eaton, 1971; Challis *et al.*, 1973) reported that large follicles generally have higher steroid contents than small follicles and the appearance of new large follicles may be reflected in the rise in serum estradiol 17-β by days 4 to 6 after ovulation. Moreover, Mills *et al.* (1981) postulated a relationship between the estrogen secretion and the wave of follicle

growth occurring in the first 6 days after mating. Thus, a pattern of increasing estrogen secretion is supposed to occur concomitantly to the increasing follicle size reported in the present study. In particular, the temporal aspects of the reappearance of large steroidogenically active follicles at day 6 of pseudopregnancy may be crucial to the survival of the developing CL.

In pseudopregnant rabbits, luteolysis normally begins on day 14 and is completed around day 18 when blood progesterone declines to basal values (Lytton and Poyser, 1982). Several different luteolytic and luteotropic forces at work as well as many levels at which these opposing and balancing influences may exert their action in the CL (Boiti, 2004). Besides prostaglandin F2α (PGF2α), other factors have recently been recognised to regulate, via paracrine and/or autocrine mechanisms, the life span of CL of rabbits, from formation to regression (Niswender *et al.*, 2000). In particular, convincing evidences suggest that the nitric oxide (NO)/NO synthase (NOS) system is deeply involved in the regulation of rabbit CL function (Gobbetti *et al*., 1999; Boiti *et al*., 2004).

Estradiol is known to be essential for the survival of the rabbit CL, prompting Hilliard (1973) to refer to estradiol as the "ultimate luteotropin" in this species. The rabbit CL becomes an estradiol-dependent tissue by day 6 of pseudopregnancy as reported by Miller and Keyes (1978). These authors demonstrated that adeguate estrogen at day 6 of pseudopregnancy is critical to the continued development of the CL. The luteotropic estrogen is supplied by the large follicles present in the ovary and destruction of these large follicles leads to immediate failure of the CL and termination of pseudopregnancy (Keys and Nalbandov, 1967; Rennie, 1968).

In the present study the ovarian ultrasound scanning allowed to describe the follicle population changes during early pseudopregnancy. The results show a depletion of large follicles present at 2 days being ≥2.0 mm in diameter. A wave of follicle growth then occurs, resulting in 71% of the follicles present at day 6 classed as large in size. The large follicles lost at ovulation have apparently been replaced by the time of luteal estrogen dependence at day 6 of pseudopregnancy. It seems unlikely that this wave of follicle growth can be explained only on the basis of the normal growth cycle of follicles in the rabbit ovary, being the experimental animals not expected to be synchronized at the time of hCG injection. A plausible explanation may lie in the pattern of endogenous gonadotropin release in the rabbit. In particular, the second surge of FSH, occurring at 24-48 h after mating, may be responsible for stimulating the follicular replacement by day 6 (Mills *et al.*, 1981).

Thus, the temporal aspects of the reappearance of large steroid-secreting follicles is in keeping with the concept that these follicles may be responsible for the rise in serum estradiol 17-β which allows continued luteal growth. There is evidence in this species that estrogen, rather than hastening the onset of luteolysis, actually has a luteotropic action in the CL, possibly by protecting it against PGF2α (Gutknecht *et al.*, 1972), or by changing the secretion of PGF2α by the endometrium from an endocrine to an exocrine mode, as proposed in the sow (Moeljono *et al.*, 1977).

By far, the most studied luteolytic mechanisms in rabbits are those associated to exogenous PGF2α administration for the simple reason that any change in blood hormone concentrations is immediately apparent (Boiti, 2004). The underlying mechanisms that control the life span of CL, however, are still not completely clear. The results presented in this report, demonstrating that the rabbit ovary is depleted of large follicles following ovulation but that large, steroidogenically active follicles are again present by day 6, could be considered as a preliminary step to understanding what mechanisms protect the CL from luteolysis until day 6 of pseudopregnancy, when CL shift from complete refractoriness to partial and complete responsiveness to PGF2α treatment. (Boiti *et al.*, 1998).

By performing the transabdominal real-time ultrasonography in the present trial, sequential monitoring of dynamic changes in the rabbit follicular population has been feasible *in vivo*. This non-invasive technique was found relatively simple, effective and ultrarapid, since the ultrasonic image facilitates immediate interpretation in most circumstances. Indeed, the ultrasound scanning could represent a rapid and reliable method for studying several reproductive functions also in the rabbit.

CONCLUSIONS

Further studies are necessary to clarify the ovarian follicular growth patterns and the overall dynamics involved in the luteolytic process. As a matter of fact, these researches may stand for a crucial key to understanding the P+ syndrome etiology. Since little is known about what factors effectively trigger luteolysis in normal, physiological conditions as well as what mechanisms protect CL from luteolysis in the first days of pseudopregnancy, the innovative and non-invasive approach supplied by the real-time ultrasonography could represent an additional tool for investigation.

ACKNOWLEDGEMENTS

This research was supported by F.A.R. 2006 of University of Sassari.

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