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RESEARCH ARTICLE

Follicular development in pregnant cows after the administration of equine chorionic gonadotropin (eCG): A new insight

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Abstract
The follicular development in the cow occurs in a wave-like pattern, and it takes place also during pregnancy. In the cow, Equine Chorionic Gonadotropin (eCG) is used for superovulation, but a decrease in total fertility has been reported, likely because of its immunogenic properties in species other than equine. In this regard, immune response has been implicated in follicular growth, ovulation, and placental development. So, aims of our study are to test the safety of eCG administered during pregnancy and characterize the ovarian activity, the quality of oocytes, the hormonal status, and interleukin levels in eCG-treated pregnant cows.

Keywords: *Cow; pregnancy; equine chorionic gonadotropin; follicular development; interleukins*

Introduction

In the cow, it is well known that the growth of ovarian follicles occurs in a wave-like fashion,[1] and during each estrous cycle, two to four waves have been reported to develop.[2–4] Each follicular wave starts with the recruitment of a pool of small follicles, proceeds with the selection of follicles that keep on growing, and ends with the dominance of a single follicle, the others becoming atretic. These three phases are characterized by slight changes in the hormonal milieu, in the size and number of follicles, and in the receptorial arrangement within the follicular wall.[5,6]

Follicular waves develop not only during the estrous cycle, but also during the prepubertal period,[7] throughout pregnancy[8] and in the early postpartum.[9] Many studies[2,10–13] have documented the existence of an ovarian follicular activity in pregnant cows independently of the stage of pregnancy, thus suggesting that there is always an intrinsic potentiality for the ovary to work, notwithstanding the degree of fetal development.

Stimulation with exogenous gonadotropins has been used to better characterize the regulation of follicular growth and development in mammals, as well as to induce superovulation in cows.[1,14–17] Equine Chorionic Gonadotropin (eCG) is still used to induce follicular growth and superovulation in domestic ruminants, considered its pronounced Follicle Stimulating Hormone (FSH)-like activity in these species.[18] The increase in follicular growth and ovulation rate elicited by eCG treatment is due to different mechanisms, including enhanced recruitment of small follicles[19] increased growth rate, and block or reversal of atresia.[20,21] eCG is a heavily glycosylated (45% w:w) dimeric glycoproteic hormone produced by mare's endometrial cups.[22] In species other than equine, this glycoprotein behaves as an heterologous protein, thus stimulating an immune response. Since now, anti-eCG humoral immune responses have been demonstrated in the plasma of treated ewes,[23] goats,[24,25] and cows,[26] and it has been correlated with a decrease in fertility.[27]

On the other hand, some evidences suggest that the immune system plays a key role in the regulation of the ovarian function. Leukocytes are important for the cyclic remodeling of the ovary due to their ability to secrete many inflammatory and immunomodulating

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molecules,[28,29] such as chemokines, involved in folliculogenesis, ovulation, development, and regression of the corpus luteum.[23,30–34]

Moreover, molecules having an immune function modulate implantation and placental growth and the interaction between the endocrine and immune systems is also implied in the feto-maternal crosstalk.[35,36]

The stimulation of lymphocytes by the conceptus, for example, may promote its own growth by increasing concentrations of cytokines at the feto-maternal interface.[37] Uterine lymphocytes and macrophages are reduced between early and mid-gestation in cattle[38] and sheep.[39]

The immune response can involve either T-helper (Th)-1 or T-helper (Th)-2 cells: Th1 cells produce mainly pro-inflammatory cytokines [such as interleukin (IL)-2], whereas Th2 cells tend to produce anti-inflammatory cytokines, such as IL-4, IL-5, IL-6, and IL-10.[40] On these grounds, we reasoned that eCG may influence the ovarian follicle development and pregnancy outcome, also affecting the production of cytokines.

On the basis of the above-mentioned elements, aims of our study were to test if eCG administration during midgestation can impair pregnancy prosecution and outcome, and to characterize the ovarian activity, the follicular development (size and number of follicles), the quality of oocytes, and the changes in hormonal status (progesterone and estradiol-17β), in pregnant cows, after the administration of a single dose of eCG. Moreover, possible changes in serum concentration of IL-2 and IL-4 following eCG administration will be evaluated.

Materials and methods

Animals and experimental protocol

The study was performed on 20 pregnant Holstein Friesian cows (3–6 years old, mean weight 600 kg), with no previous anamnesis of any kind of reproductive or calving-associated problems, and found to be healthy on clinical examination. The animals were bred on farms in Puglia, southern Italy. The average milk production was between 8200 and 8400 kg. The cows were tethered in tie stalls and fed hay, concentrate and mineral supplementation. Access to water was ad libitum.

All cows were previously bred using artificial insemination approximately 12 hrs following the onset of standing estrus. Only cows at about 100 days of gestation were enrolled in our study. Pregnancy status and gestational age were determined considering the post-artificial insemination days, by clinical examination and transrectal palpation, and with a B-mode real time ultrasonographic examination (SonoAce Pico Mycolor 202, Medison Co., South Korea), using a scanner equipped with a 7.5 MHz linear array (Medison Co., South Korea).

Cows were randomly divided into 2 groups of 10 subjects each:

- -**Group A (treated)**, administered I.M. 2000 I.U. of eCG (Folligon, Intervet Italia, Milan, Italy) (total volume 10 mL) per subject at T_0;
- -**Group B (control)**, administered I.M. 10 mL of saline solution (0.9% NaCl) at T_0 per subject.

Blood sampling

Blood samples were collected from the coccygeal vein, by venous puncture, employing pre-refrigerated vacutainer tubes, which were maintained at a temperature of +4°C and taken to the laboratory in a mean time of 30 minutes.

Samples were collected just before the treatment (T_0), and 3, 7, and 21 days after treatment (respectively, T_{+3}, T_{+7}, and T_{+21}).

Once in the laboratory, the tubes were centrifuged at 1600x*g* for 10 minutes at +4°C. The sera obtained were stored in eppendorf at –20°C, until analytical determination of progesterone and estradiol-17β. Quantitative determination of serum progesterone (P_4) concentrations was conducted with a competitive immunoenzymatic colorimetric method (Progesterone EIA WELL, Radim S.p.A, Italy). The cross-reactions of P_4 and other steroids in the serum P_4 assay are reported as follows: P_4 100%; 11-α OH-progesterone 18%; 17-α OH-progesterone 16%; 20-α OH-progesterone 1%; estradiol $< 1 \times 10^{-2}$%; testosterone $< 1 \times 10^{-2}$%; cortisol $< 1 \times 10^{-3}$%; cholesterol $< 1 \times 10^{-3}$%.

The detection limit of the assay was 0.16 nmol/l. The intra-assay and inter-assay precision had coefficients of variation of 2.9% and 4.8%, respectively. Estradiol-17β (E_2) concentration was determined by an immunoenzymatic method (Estradiol ELISA, Dia. Metra S.r.l, Italy). The cross reaction of the antibodies employed are reported as follows: E_2: 100%; Estrone 2%; Estriol 0.39%; Testosterone 0.02%; Cortisol $< 7 \times 10^{-3}$ %; Progesterone $< 3 \times 10^{-4}$ %; Dhea-s $< 1 \times 10^{-4}$ %. The lowest detectable concentration was 15 pg/mL at the 95% confidence limit. The intra-assay and inter-assay variations were 7% and 10.4%, respectively.

In 3 cows of group A and 3 cows of group B we also evaluated the serum concentration of IL-2 and IL-4 just before the treatment (T_0) and 48 hours later (T_{+2}). IL-2 and IL-4 determination was conducted with the Multi-Analyte ELISArray Kits (SuperArray Bioscience Corporation, D.B.A., Milan, Italy). Into each well of ELISArray plate 50 μl assay buffer was added and 50 μl of samples and control samples were then transferred into

the appropriate wells. Plate was incubated for 2 hours. After that, wells were washed three times. Subsequently, 100 μl Detection Antibody Solution were added and the plate was incubated for 1 hour with three washes. In the next step 100 μl Avidin–HRP were added and incubated for 30 minutes. After four washes 100 μl Development Solution were added and incubated for 15 minutes in the dark. Finally, 100 μl Stop Solution were added. The plate was read photometrically at OD 450nm within 30 minutes. Test sensitivity was 0~2.50.

Ultrasound scanning

Beginning from 2 days before treatment, and every other day for the following 22 days post-treatment (PT), ovaries of all experimental animals were examined transrectally with the above mentioned scanner, always by the same experienced operator. Briefly, after removal of feces and the introduction of the probe, ovaries were easily localized and scanned in several planes, to facilitate the visualization of small follicles (that could be otherwise masked by larger follicles or a corpus luteum), and in order to obtain images with the greatest cross-sectional areas of both follicles and corpora lutea.

For both ovaries, the number and diameter of follicles of at least 5 mm were recorded and mapped in order to facilitate tracking over consecutive days. The diameter of follicles and corpora lutea was measured using a built-in caliper, after freezing the image with the largest cross-sectional area.

Size (mm) and number of follicles were recorded. Likely modifications in the structure of gravidic corpus luteum, as well as in the appearance of new-formed corpora lutea, were also recorded. Uteri, as well the concepti, were also scanned in order to exclude any abnormal finding indicating pathological conditions or fetal distress.

Statistical analysis

All values were expressed as Mean ± S.D. Data were analyzed using Students' *t* test and one-way ANOVA, followed by Tukey-Kramer post-hoc test. A $p < 0.05$ was considered statistically significant.

Results

eCG administration had no side effects in either the cows or on the fetuses. Pregnancy prosecution was uneventful, and on the follow up, all cows gave birth to normal viable calves at term, with no calving problems. The first important evidence of the present trial was that gonadotropic stimulation induced ovarian modifications with follicular development in pregnant cows.

Figures 1–3 show the ovarian ecographic morphology before and after treatment with eCG. Ultrasound scanning demonstrated an increase in the size of both ovaries, up to two-fold the size before treatment. Ovarian enlargement was observed as soon as 24–48 hours after treatment. This increase was related to the numerical and volumetric accretion of the follicles and with the enlargement and new formation of corpora lutea. A different response was observed between left and right ovaries, both regarding the time needed for follicle development, and the total number and volume of the follicles present: a greater and faster increase in ovarian size was observed in the ovaries lacking the corpus luteum. In these ovaries, the presence of at least 3 (maximum 5)

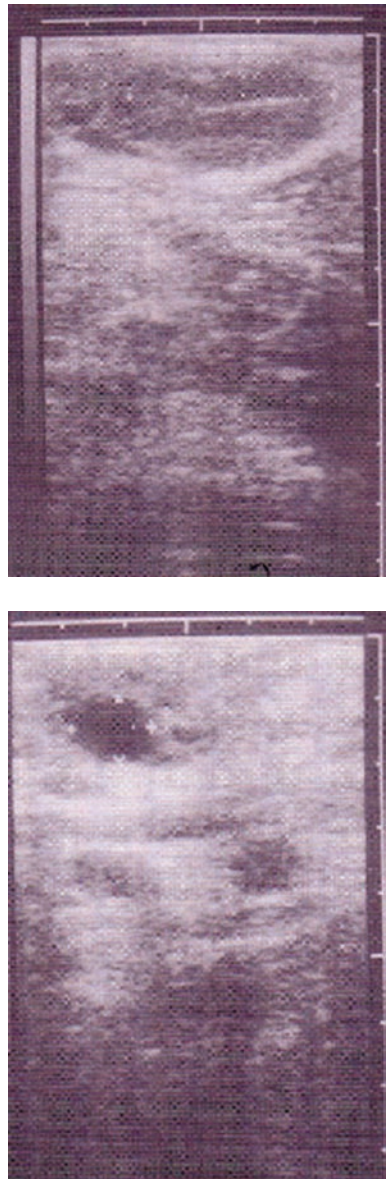

Figure 1. Ovarian ecographic morphology before treatment (T_0) in two subjects. In the upper panel a corpus luteum is evident. A follicle of 1 cm diameter appears in the lower panel.

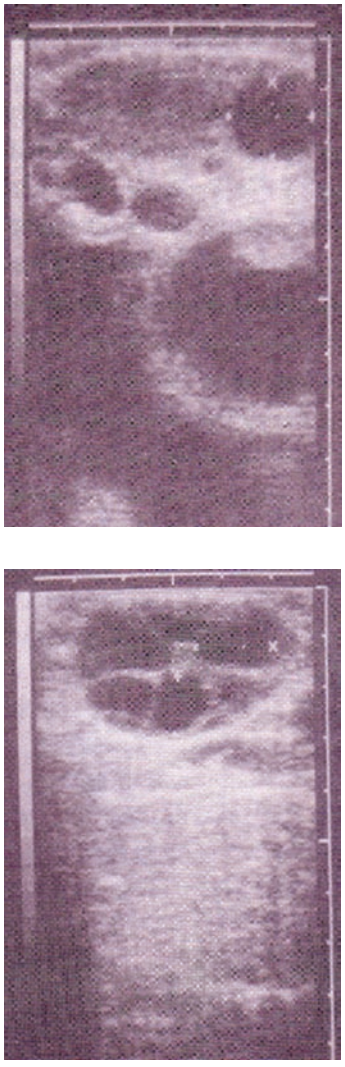

Figure 2. Ovarian ecographic morphology at T_{+4} in two subjects of Group A. Evident increase in follicular size and number.

follicles, 9–15 mm in diameter was constantly noted. Moreover, ovarian response (increase in size and follicular development), was considerably greater in the younger cows.

The ultrasonography performed between 4 and 10 days PT revealed follicles 15–21 mm in diameter. On day 12, follicular diameter began to decrease, even if new corpora lutea of small size were present. Beginning from day 4 PT, one could observe an increase in size of the gravidic corpus luteum.

Gravidic corpus luteum returned to its pre-treatment size on about the 16th day PT.

The maximum ovarian size was reached 6 to 8 days PT; subsequently, a regression was observed, with the ovary returning to its pretreatment volume on the 20th – 22th day PT. Follicular growth did not induce any heat manifestation. None of the cows of Group B showed any increase in ovarian size or follicular development.

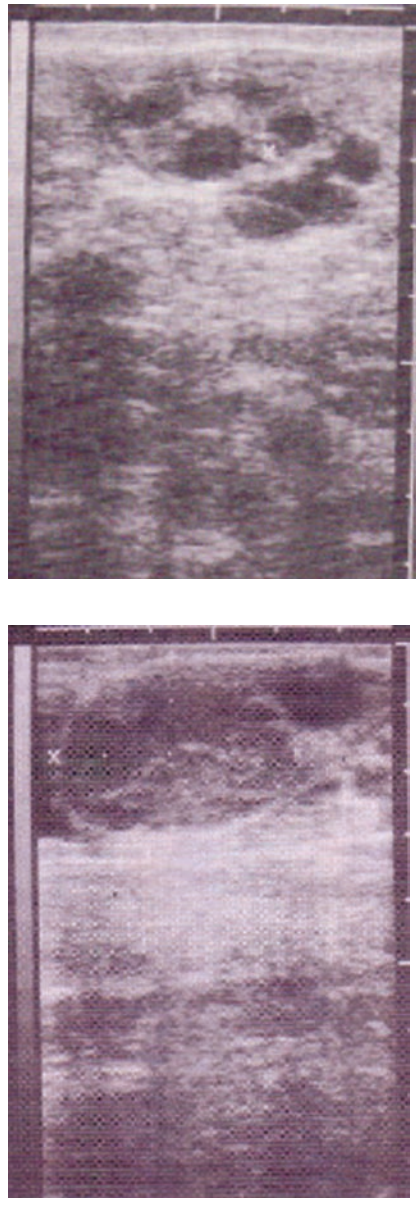

Figure 3. Ovarian ecographic morphology at T_{+12} in two subjects of Group A. An increase in the number of follicles and luteinized tissue is evident in the upper panel. The image in the lower panel shows an increase in corpus luteum size and the development of a follicle.

Table 1. Concentrations of Progesterone (Mean ± S.D.) in Group A and Group B.

Progesterone (nmol/L)	Group A	Group B
T_0	25.18 ± 6.83	25.43 ± 7.30
T_{+3}	22.77 ± 6.80	26.51 ± 6.70
T_{+7}	28.68 ± 7.21	27.7 ± 8.30
T_{+21}	24.17 ± 4.01	25.14 ± 7.20

Concentration of progesterone and estradiol-17β are shown in Table 1 and Table 2, respectively. For both the concentrations of progesterone and estradiol-17β no significant differences among the considered times

Table 2. Concentrations of Estradiol-17β (Mean ± S.D.) in Group A and Group B.

Estradiol-17β (ng/mL)	Group A	Group B
T_0	0.09 ± 0.01	0.09 ± 0.02
T_{+3}	0.09 ± 0.02	0.08 ± 0.01
T_{+7}	0.08 ± 0.01	0.09 ± 0.02
T_{+21}	0.1 ± 0.02	0.09 ± 0.01

Table 3. Concentrations of Interleukin-2 and Interleukin-4 in Group A and Group B.

		Group A			Group B		
		Cow 1	Cow 2	Cow 3	Cow 1	Cow 2	Cow 3
IL-2 (pg/mL)	T_0	0.073	0.059	0.066	0.023	0.054	0.073
	T_{+2}	0.178	0.068	0.091	0.129	0.121	0.098
IL-4 (pg/mL)	T_0	0.040	0.036	0.033	0	0.042	0.057
	T_{+2}	0	0	0.061	0.023	0	0.067

were seen, either in group A, or in Group B. IL-2 and IL-4 concentrations are illustrated in Table 3. Neither IL-2 nor IL-4 exhibited any significant difference within the group or between groups. Moreover, in some samples, levels of IL-4 were undetectable.

Discussion

eCG administration did not impair pregnancy prosecution. P_4 concentrations, in fact, did not fall below the physiological range needed for pregnancy maintenance and, even better, eCG administration was followed by a transient slight increase in its concentrations. This increase can be ascribed to the increased size of the gravidic corpus luteum.

Many other authors already described the ovarian follicular development, in pregnant cows.[2,9,13] However, there are no reports illustrating the effects of eCG during pregnancy. In our study, follicular development following eCG administration occurs in a similar way as in superovulated non pregnant cows, that is, it cannot be observed either an inhibition of follicular development, or the presence of a dominant follicle, as it happens in normal cycling cows.

Treatment elicited the formation and accretion of numerous follicles, and some of them underwent luteinization. The gravidic corpus luteum increased in size, too. Its presence did not hinder follicular accretion; however, follicles were mainly observed on the ovary lacking the corpus luteum, thus suggesting that the corpus luteum may represent a local physical obstacle for the follicular development and an endocrine/paracrine influence may regulate the distribution of the ovarian functional structures. One can hypothesize that the uterine horn ipsilateral to the corpus luteum may secrete substances locally transferred to the ovary, impairing mitosis of the follicular cells and/or antral fluid formation, enhancing apoptosis, etc.

The increased follicular growth elicited by eCG is due to different mechanisms, including enhanced recruitment of small follicles,[19] increased growth rate, block or reversal of atresia.[20,21] Apoptosis is an important process in follicular development, regulating growth or demise of recruited follicles, to ensure the selection of fewer follicles and the dominance of a single follicle.[41,42]

Furthermore, since eCG is a heavily glycosylated heterologous protein, it can be postulated that in the follicular development we observed an immune response might also be implicated. Immune cells are known to play a key role in the mechanisms of the ovarian follicular development. Resident leukocytes are now recognized to be not only passive bystanders, because they are implicated in the events leading to the cyclic ovarian remodeling, mainly through the secretion of inflammatory and immunomodulating molecules, such as cytokines.[28–34,43] These products participate to folliculogenesis, ovulation and corpus luteum formation and regression.[28,30–32]

Moreover, Wu and coworkers (2004)[43] postulated that macrophages located in the theca of growing follicles can promote cell proliferation, stimulate follicular growth and prevent apoptosis. Furthermore, ovarian macrophages secrete certain factors known to regulate follicular development, including cytokines.[44]

Given the above-mentioned statements, we hypothesize that eCG may have promoted follicular growth also by inducing a slight immune response, at least at the ovarian level, thus eliciting tissue remodeling and follicular accretion. On the other hand, untreated cows did not show any evident ovarian enlargement. Nonetheless, this slight inflammatory response has not been strong enough to induce any damage to the prosecution of pregnancy. Probably, eCG has mainly induced a type 2 immune response, known to be present during normal pregnancy.[45–47] Type 2 response is associated with preferential production of antiinflamatory cytokines (IL-4, IL-5, and IL-10).

However, according to the limited number of cows used in this study no evidence of immune involvement is evident also because we have evaluated peripheral blood mononuclear cells. Therefore, further investigations are needed, enrolling a higher number of cows, in different stage of the estrous cycle and pregnancy, trying also to evaluate local immune response status (e.g., cervical mucus and/or vaginal lymphoid tissue).

Treatment elicited a transient increase, even if not significant, in progesterone concentration, without increasing the concentrations of estradiol-17β, notwithstanding the follicular development. The failure in estradiol-17β increase in spite of the follicular development, together with the reported augmentation of progesterone concentration, suggest that the different

enzymatic milieu characterizing pregnancy, may divert the "classic" steroidogenic pathways observed in cyclic cows.

Our study shows that the ovarian activity may be independent of the endocrine balance of pregnancy, thus permitting an exogenous stimulation of the ovary. So, it is evident that, at least at the considered gestational age, there is not an absolute inhibition of the ovarian activity, but this latter is the consequence of a mutated equilibrium of the hormonal status. The circulating P_4 concentrations observed in our study, in fact, do not inhibit the development of new follicles, containing oocytes of satisfactory characteristics.

Concluding, the results of our study show that it is possible to influence the endocrine and enzymatic function of the feto-placental unit, in the pregnant cow, by the administration of FSH-like hormones. The follicular development and the physiologic prosecution of pregnancy observed in the cows administered eCG suggest a possible role for the pregnant cow, as a donor of oocytes. Moreover, a new hypothesis can be pointed out, that is, eCG can elicit follicular development also triggering a local immune response which, however, needs to be evaluated.

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