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### Detection and distribution of oestrogen receptors and progesterone receptors in leiomyomata cells

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Uterine leiomyomas are the most frequent gynaecology tumours in reproductive age women. However, their pathogenic characterization has not been totally identified.

Clinical data indicate the influence of sexual steroids in its development. The neoplasia transformation of myometrium to leiomyoma looks to involve somatic mutation of the normal myometrium, including complex interaction of the sexual steroids and local growth factors (Reimer *et al.* 2000). Historically, oestrogens have been considered the inductor of the growth of myoma. However, clinical, biochemical and histological evidence indicates an important role for progesterone (De Leo *et al.* 2002).

Results from *in vitro* studies indicate an increase of proliferating cell nuclear antigen (PCNA) in progesterone-induced leiomyoma but not in normal myometrium. (Maruo *et al.* 2000).

In this work, we showed the cellular and subcellular location and distribution of both the progesterone and oestrogen receptors in different myomatosis uterus examples. We employed 10% buffered formaline-fixed and paraffin-embedded material from biopsy specimens obtained after clinical hysterectomies. Adjacent 3 µm sections cut in order to compare the distribution of different receptors. The results are as follows.

The ER  $\alpha$  (oestrogen receptor  $\alpha$ ) can be detected in all cases assayed: both in muscle, fibroblast and endothelial cells. The ER  $\beta$  (oestrogen receptor  $\beta$ ) can be detected only in some muscle cells and ever in a nuclear location. In muscle cells, the ratio PR (progesterone receptor) to ER was, in all the cases assayed, higher than 1. In the case of ER, this ratio showed a variable distribution. PR were, in all the cases, located in the nucleus of any cell type. Histological staining clearly indicates that the nuclei of tumour cells are bigger than normal.

The characterization of PR subtypes and their location in the myoma cells, as well as the relation with the ER, will be a useful way to understand the role of the sexual steroids in the growth and development of this type of tumour. The molecular approach to the molecular bases of these relations will be a valuable tool for the discovery of an efficient and non-aggressive cure.

De Leo V (2002). *Drug Saf* 25, 759–779.

Maruo T *et al.* (2000). *Steroids* 65, 585–592.

Reimer T *et al.* (2000). *L Mol Endocrinol* 24, 33–41.

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All procedures accord with current local guidelines.

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### Influence of epidermal growth factor on cortical granule migration and steroid secretion during equine oocyte maturation

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Mammalian oocyte maturation depends on the interaction of many factors, and enables oocyte development to reach the metaphase II stage. Among others, steroid and growth factors work together in the regulation of signals associated in the oocyte maturation event. In addition, epidermal growth factor (EGF) has been shown to have positive effects during oocyte *in vitro* maturation in several species. This study aimed to establish the capacity of equine oocytes to undergo nuclear and cytoplasmic maturation in the presence of EGF and to describe the influence of EGF on steroid secretions (progesterone, oestradiol, testosterone and androstenedione) following *in vitro* culture.

Ovaries were obtained from horses that had been humanely killed. Cumulus-oocyte complexes ( $n = 204$ ) were obtained by aspiration and subsequent scraping of equine ovarian follicles, and were matured in TCM 199. They were then cultured in two different treatment groups: control and EGF (50 ng ml<sup>-1</sup>). Each group was pretreated with either 0, or 10<sup>-6</sup> mM tyrphostin A-47, a specific tyrosine kinase inhibitor. At the end of maturation, medium samples were collected for steroid determinations by using EIA. This procedure was as previously validated by Lorenzo *et al.* (1997). Nuclear maturation was determined as the percentage of oocytes reaching the metaphase II stage at the end of the culture period. Cytoplasmic maturation (measured as cortical granule (CG) migration and distribution) was determined by fluorescein isothiocyanate-labelled *Lens culinaris* agglutinin with laser confocal microscopy. All statistical comparisons and analysis were done by using ANOVA, using Catmod procedure of SAS.

Results show that treatment with EGF significantly increased ( $P < 0.05$ ) the incidence of metaphase II stage when compared with the control group (71.6 versus 32.1% in controls). When oocytes were cultured with EGF, all the matured oocytes showed complete CG migration to the cortex, to form a continuous monolayer under the oolemma but no CG-free domain was observed in oocytes during maturation. The specific tyrosine kinase inhibitor A-47 was effective in suppressing nuclear and cytoplasmic EGF-induced effects on oocytes cultured with EGF. In addition, compared to controls, EGF supplementation in the maturation media was associated with a significant increase of progesterone and androstenedione ( $P < 0.01$ ), and positive correlations between oestradiol/androstenedione ratios and nuclear maturation were found. These observations together with the increase of nuclear maturation rates, and complete CG migration to the cortex when using EGF in culture media, suggest an important role for EGF in the regulation of equine oocyte maturation.

Lorenzo PL *et al.* (1997). *J Reprod Immunol* 35, 11–29.

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### Neonatal changes in the phenotype of lamina propria lymphocytes in the small intestine of Lewis rats

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During the early neonatal life, important changes occur in the gut. Challenges from milk and the microbial flora conduct mucosal immune system maturation. Lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL) constitute the non-aggregated gut-associated lymphoid tissue (GALT) acting as effector cells. Although it has been described that LPL are phenotypically and functionally similar to peripheral lymphocytes, little is known about the ontogenesis of this GALT population in the rat.

The aim of the present study was to establish the phenotypic maturation of LPL in suckling Lewis rats, from day 1 up to day 21 after birth. The study was in compliance with the guidelines for the care and use of laboratory animals approved by the Ethic Commission for Animal Experimentation of the University of Barcelona. Rats were killed by decapitation. LPL were isolated from the small intestine by previously removing IEL and subsequent enzymatic digestion. Phenotype was assessed by FACS analysis, using double labelling techniques and monoclonal antibodies for detection of lymphocytes (CD45+), B cells (CD45RABC+, IgA+ or  $\kappa$  chain+), T subsets (CD4+, CD8+, CD5+, CD3+, TCR $\alpha\beta$ +), and NK cells (NKR-P1+). The surface expression of activation and adhesion molecules (CD25, CD90 and CD2) was also determined.

CD45+ LPL (total lymphocytes) showed a progressive increase in percentage with the age of animals being more than 80 % of gated lymphocytes from day 14. In this CD45+ population, the highest percentage corresponded to CD8+ LPL (50–60 %) during the first two weeks. B cell proportion increased up to more than 35 % just before weaning. CD4+ LPL percentage showed a positive evolution during the studied period. Most of CD4+ LPL cells were CD5+, CD3+, CD2+ and TCR $\alpha\beta$ +, although proportions varied along suckling period. About 20–30 % of CD4+ LPL were also positive for CD90. In addition, there were 20 % of CD4 LPL that also expressed CD25. CD8+ LPL cells include lymphocytes bearing CD8 molecule as the homodimer  $\alpha\alpha$  or the heterodimer  $\alpha\beta$ . Most of the CD8 T cells were CD8  $\alpha\alpha$  until day 11, but later the ratio  $\alpha\alpha/\alpha\beta$  tended to be 1. Moreover, most of CD8+ LPL were CD5–, TCR $\alpha\beta$ + and CD3+ although proportions varied along the suckling period. NK cells represented about 20 % of LPL on day 14, and decreased thereafter. Only a half of NK LPL shared CD8 positivity. In summary, this study shows that the LPL phenotype does effectively change during the suckling period when the immune system is still maturing.

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*All procedures accord with current local guidelines.*

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### Evolution of spontaneous immunoglobulin-secreting cells in spleen and gut-associated lymphoid tissue during the suckling period in rats

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Although immune system development seems partly regulated by internal triggers, its complete maturation appears to occur only after the intestine is challenged postnatally with both microbial and nutritional antigens. The aim of the present study was to investigate the immunoglobulin (Ig) secretion ability of cells from gut-associated lymphoid tissue (lamina propria lymphocytes, LPL) and spleen during the suckling period of Lewis rats.

The study was in compliance with the guidelines for the care and use of laboratory animals approved by the *Ethic Commission for Animal Experimentation* of the University of Barcelona. Spleen and small intestine were removed from decapitated rats aged between 1 and 21 days, and also from 9-week-old animals to be used as controls. Splenic cells were obtained by pressing the spleen through stainless-steel sieves and isolation with a Nycoprep gradient. To obtain LPL, the small intestine was treated with DTT-EDTA to remove intraepithelial lymphocytes. Then, the remaining tissue was enzymatically treated and cells were purified by Percoll gradient. Splenic cells and LPL were assayed for the spontaneous secretion of IgA, IgM, and IgG by enzyme-linked immunospot techniques (ELISPOT) using specific mouse anti-rat isotypes monoclonal antibodies (MoAb) coated to nitrocellulose plates. Cell suspensions were incubated for 20 h and later, Ig secretion was evaluated by subsequent addition of biotinylated anti-isotype MoAb, extravidin-peroxidase, and a peroxidase substrate. Spots were counted by an ELISPOT reader.

In spleen, a few IgG-secreting cells could be quantified along the suckling period and they decreased in adult rats. The number of IgM-secreting cells increased in spleen during the first 3 weeks of life, and diminished in adult rats. The number of IgA-secreting cells rose throughout the suckling period until adult age. The study in LPL revealed that there was no Ig production until day 14. From then, IgM and IgA production could be quantified. The number of IgM-secreting cells was higher than that of IgA from day 14 to day 21. In contrast, in adults, intestinal IgA-secreting cells were more abundant than IgM-secreting cells. Gut spontaneous production of IgG was not observed.

In conclusion, we observed an earlier Ig secretion in spleen than in LPL, the splenic IgG and IgM secretion being higher during the suckling period than in adult rats. With regard to LPL, during the first weeks of life, the IgM secretion prevailed over IgA secretion but this proportion shifted in adult rats which would correspond to the main levels of IgA in intestinal lumen.

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**Long-term consequences of diabetes during pregnancy and lactation**

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Maternal diabetes during pregnancy still carries a risk of malformed and spontaneously aborted offspring. Besides, recently there has been obtained epidemiological evidence for the long term effects on adult health of altered 'programming' during a sensitive period of early life, where stimulus or insult at a critical, sensitive period of early life has permanent effects on structure, physiology and metabolism. Since besides intrauterine hyperglycaemia, the offspring of a diabetic mother could be affected by altered lactation as a consequence of the known decreased milk yield of the mother, the present study has addressed the long term consequences of changes in the suckling schedule of newborns of diabetic rats.

At day 8 of pregnancy, some rats were made diabetics by treatment with 5 mg of streptozotocin (STZ)/100 g b.w. (D), whereas controls (C) were treated with medium (50 mM citrate buffer, pH 4.5). At the time of delivery the litters were unified to five pups per dam and they were distributed among four groups as follows: (i) pups from C mothers that were allowed to suckle from C dams (cC); (ii) pups from D mothers that were allowed to suckle from C dams (dC); (iii) pups from C mothers that were allowed to suckle from D dams (cD); and (iv) pups from D mothers that were allowed to suckle from D dams (dD). Pups were weighed at birth and at 20, 30 and 60 days of age. At 60 days of age, an oral glucose tolerance test (OGTT) was performed in all the pups, as follows: blood was collected from the tail (time 0), and rats received an oral load of 2 g glucose per kg b.w., blood being also collected from the tail 15, 30 and 60 min thereafter, and glucose, insulin, triglycerides (TG) and free fatty acids (FFA) were measured in plasma aliquots. At the end of the experiments, all animals were humanely killed. Statistical analysis was performed by ANOVA or the Mann-Whitney test as appropriate.  $P < 0.05$  was considered significant.

At birth, dC and dD pups weighed less than cC and cD pups. Besides, whereas dC pups completely recovered their body weight, attaining values that did not differ to cC, the increase of body weight and size of cD pups were lower than in cC, attaining statistically significant differences. In dD pups, the increase in both plasma glucose and insulin in the OGTT were smaller than in any of the other groups, whereas in dC pups basal glucose (time 0) was higher and in cD plasma insulin at 0 and 15 min time was lower than the other groups but plasma glucose did not differ.

Thus although the present findings show long term consequences of the intrauterine diabetic milieu on growth and in the glucose/insulin axis, they also show that changes in dietary conditions during suckling also affect these variables even in pups from control dams.

*All procedures accord with current local guidelines.*

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**Relationship between maternal feed intake, ponderal and metabolic factors with birthweight and offspring number under different dietary protein concentrations**

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The purpose of this work was to correlate the maternal feed intake, ponderal and metabolic parameters with the neonatal birth weight and the offspring number to obtain linear models that could predict dependent variables in pregnant Wistar rats and their neonates fed either 4 or 20 % dietary protein concentration. The independent variables considered were (a) maternal feed, energy and protein intakes; (b) maternal body weight before and after Caesarean section (rats were humanely killed), and maternal body weight gain before and after Caesarean section; and (c) maternal metabolic factors including plasma insulin and glucose, insulin resistance (insulin/glucose ratio), growth hormone (GH) and insulin/GH ratio.

Best-fit stepwise multiple regression analysis was used to select factors (independent variables) that could be entered in the models. Independent variables were grouped in two blocks: (a) feed and ponderal variables, and (b) metabolic variables. The statistical criterion for a variable to enter in a given model was a probability of  $F < 0.05$ . The variance found in the birth weight was explained in a 32 % by the maternal protein intake ( $R^2 = 0.32$ ) and in a 47 % by the maternal insulin resistance ( $R^2 = 0.47$ ). Maternal body weight gain after a Caesarean section was the independent variable, among the feed and ponderal parameters, that had the strongest correlation ( $R^2 = 0.26$ ) with the number of offspring. Also, the linear model obtained for the offspring number with metabolic variables included two factors: glucose and insulin/GH. In the first step, glucose entered with  $F < 0.001$  and  $R^2 = 0.33$ . In the second step, insulin/GH increased  $R^2$  to 0.44 ( $F = 0.027$ ).

In conclusion, these results support that various maternal ponderal and metabolic factors may modify neonatal growth and offspring number in pregnant rats fed 4 and 20 % dietary protein concentration. The higher predictive factors were insulin resistance for the birthweight, and both maternal plasma glucose and insulin/GH ratio for the offspring number.

*All procedures accord with current national guidelines.*