.-Abstract

Introduction:

During fetal and early postnatal development the ovaries present significant morphological changes, which are controlled hormonally. Through their specific receptors, the sex steroid hormones fulfill an important role in the differentiation and development of the reproductive system, the regulation of ovarian function and upkeep of female fertility. The term endocrine disruptor (ED) refers to those substances which have the ability to imitate or antagonize endogenous hormones. The first paper dealing with it was published in 1993 and since then, it has become obvious that the presence of ED in the environment could represent a threat to human and animal health. According to the American Agency for the Protection of the Environment, ED is referred to as a ***"substance or mix of exogenous substances which alters one or more functions of the endocrine system and therefore causes unfavorable effects on the health of an intact organism, its progeny or subpopulations."*** (USEPA, 1997).The discovery of the existence of chemical substances in the environment with agonist/antagonist activity of endogenous hormones was associated with the interest in finding out about its effects on the development and reproductive capacity in animals of the wildlife, livestock species and human beings. Despite the fact that the whole endocrine system may be involved in this hormonal disorder, the information available on hormonal imbalance caused by chemicals with estrogenic activity (known as xenostrogens) is, in quality and quantitatively, most abundant. Some EDs, which have been associated with effects on the development and reproduction, imitate the activity of 17β-estradiol (E2) and it was hypothesized that these substances may be associated with several pathologies such as breast cancer, deficiency and abnormality in the sperm count, increase in the incidence of prostate and testicle cancer and other male related reproductive dysfunctions. Most of the current evidence suggests that mammals are more sensitive to EDs during fetal and early postnatal life than in adult life. This may be due to greater sensitivity of the fetus and/or newborn during their development than during adulthood. Moreover, the endocrine mechanisms of feedback and the immune system are not completely developed yet, which may favor the appearance of adverse effects. Among most used and studied EDs we can mention two synthetic xenostrogens: BPA and DES. Unlike the interest borne to lab or wild life animals, so far little attention has been paid to the effects of EDs on animals of the livestock. DES is a powerful xenostrogen representative of chemicals with demonstrated estrogenic activity. It was widely used on pregnant women during the 50s and 60s to avoid miscarriages and pregnancy

complications. BPA (di-(p-hidroxifenil) dimetilmetano) is one of the industrial chemicals with the highest volume of production in the world. More than 3 million tons are produced annually. Every year, its production increases by 10% and more than 100 tons are released into the environment yearly. BPA is one of the most studied and investigated xenostrogen. BPA has drawn the attention of regulatory agencies and of scientists from various countries due to its estrogenic properties both in vitro and in vivo given the known role played by estrogens in the regulation of animal and human physiology and physiopathology. One of the biggest concerns related to the exposure to BPA lies in the effects that it may cause on human especially when exposed during the most sensitive stages of development: fetal, neonatal, prepubertal and pubertal. Research that has been conducted in the last few years suggests that hormonal pathologies which become obvious in women as from puberty may originate in the fetal or neonatal life when the ovaries are still being formed. In sheep, prenatal exposure to BPA has shown evidence of lower weight in the offspring at birth and finished their breeding season later during adulthood. Nevertheless, little is known about the effects of neonatal exposure to BPA or DES on the development of the sheep's ovary. The selected compounds for this study have high environmental impact, both in rural and urban areas. Note that the doses used in our experiments were similar to those determined by other studies such as those present in the environment and to which animals and humans are exposed daily.

Objectives: To determine whether early postnatal exposure to DES or BPA affects the development of the sheep's ovary and may cause reproductive consequences during adulthood. We were interested in studying whether there were any effects produced on the development of the ovary in the short run (in prepubertal animals) following the exposure to the xenostrogens and if any changes were observed in the functionality of the organ. Specifically in prepubertal female Ovis aries (domestic sheep), exposed during their first two weeks of life to the xenostrogens DES or BPA, we studied: a) ovarian follicular dynamics establishing the number of follicles in every one of the different follicular populations (primordial's, in transition, primary, small preantrals, big preantrals and small antrals); b) the expression of molecules that are associated to the cycle of cellular division, by means of detection through immunohistochemistry (IHQ) of the expression of Ki67 y p27; c) the percentage of atretic follicles and the incidence of multiovular follicles (MOFs); d) serum levels of E2 and T to analyze changes in the ovarian steroidogenesis. e) the response to a gonadotrophic treatment of follicular

superstimulation. In addition, we studied the pattern of expression of steroid receptors in the ovary of control lambs during the first month of life, in order to determine the ontogeny of REα, REβ and RA when the animals will be exposed to xenotrogens.

Materials and Methods:

In order to develop the proposed objectives, the work was divided into 3 experiments:

Experiment 1: This first experiment was designed in order to study, by IHQ, the ontogeny of the receptors of steroid hormones (REα, REβ, RA) on samples of lambs ovaries on postnatal days 1, 5, 10 and 30.

Experiment 2: After birth and identification, female lambs were randomly assigned to one of the following postnatal treatments which were administered subcutaneously on a daily basis from PND 1 (day of birth) to 14: 1) Control on those that were given corn oil (vehicle) (n=10); 2) DES (Sigma) dose: 5 µg/kg/day (n=6); 3) BPA50 (99% purity, Aldrich, Milwaukee, WI): 50 µg/kg/day (n=6) (Figure 13). On PND 30 the lambs were weighed then they underwent bilateral ovariectomy under general anesthesia. We determined the effects of the exposure to xenostrogens on the development of the ovary in prepubertal ewes, specifically on postnatal day 30 (PND 30). The ovary samples allowed us to determine: the weight of the organ, parameters that determine the follicular development (follicular dynamics, complete reserve of oocytes, incidence of MOFs and percentage of atretic follicles; expression of receptors of steroid hormones (REα, REβ, RA) by IHQ and indicators associated to the cycle of cellular division (Ki67 and p27). On PND 30, serum samples were taken from the same animals in order to quantify the circulating levels of E2 and T.

Experiment 3: It was designed to study whether the postnatal exposure to xenostrogens affects the ovarian functionality. We used female lambs that had been exposed to xenostrogens postnatally according to the experiment 2 with addition of a group of animals that had been exposed to BPA with a dose 100 times inferior to the safe dose (0,5 µg/kg/day). Later, on PND 30, all the animals were submitted to a gonadotrophic treatment of follicular superstimulation with ovine FSH. The ovary samples allowed us to determine: the number of follicles equal or greater than 2 mm, the percentage of atretic follicles and the expression of REα, REβ, RA and Ki67. In addition, serum samples were obtained at the beginning and the end of the gonadotrophic treatment in order to determine the circulating levels of E2 by means of radioimmunoassay (RIA).

Results: The experimental results that have been achieved in this thesis showed that: a) During exposure period to selected xenostrogens (PND 1-14) the lamb's ovary

expresses REα, REβ and RA in different tissue compartments: REβ and RA expressed with greater intensity while REα expression is low or null. b) Lambs neonatally exposed to DES and BPA showed a decrease in primordial follicles reserves and an increase of follicular development. c) The exposure to BPA reduced the ovarian weight and raised the incidence of MOFs. d) BPA promoted granulose and theca cells proliferation of antral follicle and increased both the number of atretic follicles and the expression of p27. e) The neonatal exposure to DES and BPA reduced the pool of primordial follicles on stimulation of its initial recruitment as well as the subsequent follicular development to the antral stage. This acceleration in the folliculogenesis in prepubertal lambs led to a greater number of atretic follicles. f) Lambs neonatally exposed to DES and BPA developed a lower number of follicles ≥2 mm and a lower number of atretic follicles in response to oFSH treatment. g) The animals exposed to DES and BPA and treated with oFSH showed a lower expression of RA in granulosa and theca cells, besides lower serum levels of E2 suggesting an alteration in the ovarian steroidogenic response. h) Taken as a whole, the results confirm the usefulness of the female lamb as suitable model to study endocrine disruption. i) Postnatal exposure to low doses of BPA and DES altered both the development and ovarian functionality in the model used.

Conclusions:

The results presented in this thesis allow us to draw the following conclusions:

- ✓ The ovaries of the control lamb group (not treated) showed a high and early expression of REβ during the first month of life. Due to the fundamental role of REβ in the ovarian development and functionality we suggest that it could be a mediator of the effects of xenostrogens on the ovary.
- ✓ Early postnatal exposure of the lamb to xenostrogens DES and BPA altered the normal development of the ovary: lower ovarian weight, decrease in primordial follicles reserves due to stimulation and acceleration of follicular development, increase of follicular atresia and increase in the incidence of MOFs.
- ✓ The doses used for the study are low and as for BPA we selected the dose defined as safe (50 µg/kg/day BPA). The effects detected, not only in this study but also in previous publications with other species, imply the need for a revision of the dose defined as safe for BPA.
- ✓ The administration via sc from birth up to PND 14 caused alterations in the ovary that were detected on day 30. As in previous studies conducted on rats and

mice, exposure during the first weeks of postnatal life in lambs represents a period of high susceptibility to the effects of EDs.

- ✓ Some of the changes detected (increase in the proliferation of granulosa cells and in the follicular activation) are similar to those described in the ovarian polycystic syndrome (SOP) of the woman. Based on this, we suggest that the model used is able to provide information about the possible etiology and increase of the incidence of this and/or other reproductive diseases.
- ✓ Early postnatal exposure of the lambs to xenostrogens followed by the stimulation with exogenous gonadotropins (on PND 30) allowed us to demonstrate functional alterations in the ovary: decreasing the number of stimulated follicles as a steroidogenic response.
- ✓ We may conclude that the sheep turns out to be a useful animal model to study the effect of the EDs and that the design of stimulation with gonadotropins is efficient to highlight functional alterations in hormone dependent tissues, such as the ovary.
- ✓ The future study of the reproductive performance of these lambs will define whether the observed alterations produce consequences during adulthood. Maintaining lambs exposed until reaching adulthood will determine whether the effects produced by the exposure to xenostrogens are organizational effects (ie, permanent).