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From Cell Biology to Tissue Engineering

Development of germ cell neoplasia *in situ* in chinchilla rabbits

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Summary. The present study was designed to describe the development of germ cell neoplasia *in situ* in Chinchilla rabbit by administration of estradiol. The study was performed in rabbits distributed into two groups: control and 17 β-estradiol. The determination of histological alterations and POU5F1 and c-kit proteins employed as biomarkers for the diagnosis of this neoplasia was carried out. Testicular descent and complete spermatogenesis were observed in the control group. The protein biomarkers were negative. However, in the rabbits treated with estradiol, the testes remained undescended with the gonocytes undifferentiated to spermatogonia. There were histological lesions owing to germ cell neoplasia *in situ* and positive to POU5F1 and c-kit proteins. These findings indicate that the chinchilla rabbit is an ideal model to study this neoplasia in which the histological characteristics and biomarkers of the disease could be clearly observed. Using this model we suggested that the persisting gonocytes could be responsible for the development of germ cell neoplasia *in situ*.

Key words: Germ cell neoplasia *in situ*, POU5F1, c-kit, Rabbit, Animal model

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Introduction

The incidence of testicular cancer (TC) has increased in different countries of the world (Purdue et al., 2005; Bray et al., 2006). This fact has been associated to exposition to compounds with estrogenic activities that act as endocrine disruptors during gestation and neonatal period and so, could have led to the development of testicular dysgenesis syndrome (TDS) comprising of pathologies like cryptorchidism, hypospadia, infertility, and TC. It has been reported that during early intrauterine development, the gonadal microenvironment is highly regulated by and sensitive to hormones and paracrine factors (Rajpert, 2006).

The germ cell neoplasia *in situ* (GCNIS), also called carcinoma *in situ* of the testis (CISt), is a pathology with a specific histological pattern that precedes the development of testicular germ cell tumors (TGCT) like seminoma and no seminoma (Skakkebaek, 1978; Müller et al., 1984). The GCNIS is characterized by the presence of atypical cells, formation of multi-layer and thickening of basal lamina, cellular proliferation, spermatogenesis arrest, movement of Sertoli cell nuclei to the center of tubular (Skakkebaek, 1972; Montironi, 2002), and positive to specific protein markers such as POU5F1, also called OCT4 (Looijenga et al., 2003) and c-kit, also called CD117 (Rajpert-de Meyts and Skakkebaek, 1994). It was suggested that the origin of GCNIS occurs during embryonary development where the gonocytes were proposed as the principal cause since their morphological characteristics, expression of pluripotential proteins like POU5F1, and survival proteins like c-kit are similar to atypical cells of GCNIS

(Skakkebaek et al., 1987; Jorgensen et al., 1995; Rajpert-De Meyts et al., 2003; Looijenga et al., 2003; Honecker et al., 2004; Hoei-Hansen et al., 2005a; Sonne et al., 2009; Hutson et al., 2013).

Knowledge of the development of GCNIS is limited because there is no animal model where the development of the pathology is closely similar to that of humans that would permit timely determination of the onset of the disease and give way to proposals of strategies to reduce the risk of the same. The models presently known have employed different species like *C. elegans*, zebrafish, mice, among others which are genetically manipulated to generate the disease (Subramaniam and Seydoux, 2003; Lee et al., 2009; Neumann et al., 2011). It has been reported that dogs spontaneously develop TGCT (Scully and Coffin, 1952) although it was classified as a spermatocytic seminoma type. However, none of these models is closely related to enviromental development of this pathology as seen in humans. For these reasons, the objective of this work was to describe the development of GCNIS in Chinchilla rabbit by administration of estradiol.

Materials and methods

Samples

Twenty-four rabbits (*Oryctolagus cuniculus*) belonging to the Chinchilla breed were used. The animals were maintained in room lighting of 16:08-h light/dark cycle and were kept in stainless steel cage (90x60x40 cm of height) with their mothers until weaned. At 40 days postpartum (dpp), they were separated and kept in individual cages with free access to food (Purina® rabbit chow) and water. All animals were humanely treated following the ethical principles and specified regulations as stated in the Official Mexican Norm NOM-062-200-1999 entitled "specifications for the production, care and use of laboratory animals". This work was approved by Institutional Animal Care and Use Committee. The animals were distributed in two groups: control (group 1) and estradiol (group 2) each with 12 animals. The control group received propylene glycol every third days from 7 to 60 dpp while each of the estradiol group received subcutaneous administration of 17 β-estradiol at 0.12 mg (Sigma-Aldrich, St Louis, MO, USA) in a solution of 50 μl of propylene glycol vehicle every third days from 7 to 60 dpp. On 60^{th}, 90^{th}, and 180^{th} dpp, 4 animals per group in accordance with age were euthanized with an overdose of sodium pentobarbital (i.p. 100 mg/kg). Three testicular tissue samples were taken from each animal and fixed with Karnovsky solution for histological analysis, immersed in paraformaldehyde for immunohistochemical determination of GCNIS protein markers, (POU5F1 and c-kit) and cellular proliferation (Ki-67, also called MIB-1), and were ultrafrozen at -70°C to confirm the results by RT-PCR.

Processing of biological material

Morphology

The testicles were dissected, weighed and washed with saline solution (0.9%). Tissue specimens were fixed overnight with Karnovsky's aldehyde solution (Karnovsky, 1965) without Ca^{2+}, pH 7.4, postfixed with 1% OsO_4, dehydrated in a graded series of ethanol and embedded in EPON 812 (Ted Pella, INC. CA, USA). Semithin 1 μm-thick sections were cut from EPON blocks using an Ultracut UCT microtome (Leica, Vienna, Austria) and stained with 0.5% toluidine blue.

The histological analysis of the seminiferous tubes was performed using a BX 51 Olympus light microscope (Tokyo, Japan). Twenty to thirty transversal sections of the seminiferous tubes per animal were analyzed. The area of seminiferous epithelium was determined by subtracting the internal area from the external area, using an image analyzing system (Image-Pro Plus 5.1, Media Cybernetics, INC. MD, USA). The maturity index of seminiferous epithelium (Johnsen index), histopathological index (Vigueras et al., 2011), gonocytes and spermatogonia numbers were determined and their assessment were carried out only in transverse sections (seminiferous epithelium cycle stage VII of the control group). The gonocytes were identified by their big size, round shape, pale cytoplasm, mitochondrial disposition around the nucleus, and by their big central nucleus which in majority of the cases were evident. The spermatogonia were characterized by their small size, round and flattened shape, central nucleus dyed with fine and thick chromatin spread or located peripherally around the nucleus.

Electron microscopy

To confirm the presence of persistent gonocytes, the testicular tissues of the rabbits were ultracut to 60-70 nm thick. Sections were stained with uranyl acetate and lead citrate, and examined with a JEM-1011 (JEOL, Osaka, Japan) microscope.

Immunohistochemistry (POU5F1, c-kit and Ki-67)

Sections in paraffin, prepared as described above, were mounted on poly-l-lysine coated slides (Sigma-Aldrich). Six sections from each testis were prepared. The slides were deparaffinized in xylene for 30 min. After rehydrating through a graded ethanol series, they were boiled in citrate buffer (pH 7.6) for 5 min in a microwave oven set at 800 W. Sections were delineated by Dako pen (DAKO, Carpinteria, CA, USA) and subjected to proteinase K digestion (Sigma-Aldrich) with 4 mg/ml of chlorhydric acid 0.01 N for 20 min. The sections were then washed with PBS and incubated in HCl 2 N for 30 min. Later, they were again incubated in sodium borate (Sigma-Aldrich) 0.1 M pH 8.5 for 10 min and washed in PBS. Slides were blocked with 1%

donkey serum albumin, (Amersham Biosciences, Buckingham-shire, UK) for 2 h, and incubated for 18 h at 4°C with a mouse-monoclonal antibody against POU5F1 (Santa Cruz Biotechnology CA, USA) at a 1:50 dilution; mouse-monoclonal antibody against c-kit (Santa Cruz Biotechnology) at a 1:50 dilution, or with a mouse-monoclonal antibody against Ki-67 (DAKO) at a 1:250 dilution to determine cellular proliferation. Slides were then incubated with biotinylated anti-mouse IgG (Santa Cruz Biotechnology) at a 1:100 dilution for 1 h and then with streptavidin-peroxidase conjugate (Rabbit Immunocruz staining system, Santa Cruz Biotechnology) for 30 minutes in accordance with the manufacturer's instructions. Sections were incubated in a peroxidase substrate solution containing 1.6 ml of distilled H_2O, 20 µl 10x substrate buffer, and 40 µl 50x diaminobenzidine chromogen (kit from Santa Cruz Biotechnology) and 1% H_2O_2 (Merck, Darmstadt, Germany) in methanol for 30 min and then counterstained with hematoxylin, dehydrated through a graded series of ethanol and cleared with xylene. All dilutions and thorough washes between stages were performed using phosphate-buffered saline (PBS) unless otherwise specified. The human seminoma and rabbit fetal testis were used as positive control to POU5F1 and c-kit.

Negative control sections were processed in an identical manner but the primary antibody incubation step was omitted. Sections were mounted with Entellan mounting medium (Merck). All tissues were processed at the same time to minimize any potential variance in labeling procedure. The histological analysis of the seminiferous tubes was performed by an observer with the help of a BX51 Olympus light microscope. Slides from different ages were randomized and coded in such a way that all subsequent analyses were conducted in a blinded manner. All histological examinations were performed by a single observer.

The number of cells positive to POU5F1, c-kit, and Ki-67 was determined in 10 000 μm^2 of testicular tissue. A total of 50 seminiferous tubules were evaluated per animal.

RNA extraction and real-time PCR

For determination of POU5F1 and c-kit by RT-PCR, 100 mg of testicular tissue was frozen and stored at -70°C. Total RNA was prepared homogenizing the tissue in 100 μl Trizol reagent and extracted according to the manufacturer's instructions (RNeasy® QIAGEN Austin, Texas USA). Reverse transcriptase-polymerase chain reaction (RT-PCR) amplifications were performed using two primers designed on the basis of what was previously reported (Table 1). For expression control, amplification of oligonucleotides used as internal controls was β actin (Pan et al., 2004; Rodriguez et al., 2007; Racila et al., 2011). The amplification reactions were carried out by means of One step RT-PCR kit (QIAGEN) under the conditions recommended by the manufacturer. cDNA synthesis was performed at 50°C for 30 min and amplification conditions were: 94°C/5 min (once) for initial denaturation, 94°C/30 sec, 55°C/30 sec, 72°C/60 sec (34 cycles), 72°C/2 min (once). In order to confirm that the amplified products were not genomic DNA in origin, we used RT-free control (Taq DNA polymerase but not template DNA added) for each RT-PCR.

Ten μl of each RT-PCR reaction were electrophoresed on a 2.5% agarose gel. The size of each of the products is shown in Table 1. RT-PCR was repeated three times for each sample.

Statistical analysis

Results are expressed as a mean ± S.E. and were analyzed using the student's t-test for independent groups comparing the estradiol groups versus the control group per age group with a $p<0.05$ considered as statistically significant.

Results

In the control group rabbits, the testes descended in the scrotal bag at around 50 dpp and the testicular/body weight relationship showed a clear increase during their development (Table 2). In the estradiol exposed group, bilateral inguinal cryptorchidism was observed in all the ages. The testicular/body weight relationship reduced by up to 70% with respect to the control group ($p<0.05$, Table 2).

Animals of 60 dpp

In the control group, the testicular gonocytes were observed with or without contact with the basal lamina. In some tubules, gonocytes could be seen among the recently differentiated spermatogonia (Fig. 1). The seminiferous epithelial area, the number of gonocytes and spermatogonia, the histopathological and epithelial maturity (Johnsen) index were within normal parameters (Table 2, Figs. 1, 2). POU5F1 and c-kit immunoreactivities were slightly evident in the few gonocytes that still persisted. The positivity of POU5F1 was nuclear and cytoplasmic (Table 3, Figs. 3, 4). Ki-67 was

Table 1. Primer sequences used for RT-PCR.

Gene name	Primer sequence	Product size (bp)
POU5F1	F 5'-CAGTGCCCGAAACCCACAC-3' R 5'-GGAGACCCAGCAGCCTCAAA-'3	161
c-KIT	F 5'-TACTCATGGTCGGATCACAAA-3' R 5'-CCACTTCACAGGTAGTCGAGC-3'	106
Beta-actin	F 5'-CACCCTGAAGTACCCCATCGAGCA-3' R 5'-CAGGTCTTT GCGGATGTCCACGTCAC-3'	685

bp: base pairs.

evident in gonocytes and spermatogonia in proliferation (Fig. 5). Due to scarce expression of POU5F1 and c-kit, it was not possible to observe the messengers in this stage of development in RT-PCR analysis (Fig. 6).

In the estradiol exposed group of animals, slight thickening and folding of the basal lamina and cells in degeneration were observed (Fig. 1). The number of gonocytes was significantly higher while that of the spermatogonia was less when compared with the control group ($p<0.05$, Fig. 2). The seminiferous epithelial area and epithelial maturity index were significantly reduced ($p<0.05$) while the histopathological index increased significantly ($p<0.05$) in comparison with the control group (Table 2, Fig. 1).

The number of immunoreactive gonocytes to POU5F1, c-kit, and Ki-67 proteins was significantly higher in comparison with the control group ($p<0.05$, Table 3, Figs. 3-5). The immunoreactivity to POU5F1 was cytoplasmic and nuclear (Fig. 3). These results were confirmed by RT-PCR (Fig. 6).

Animals of 90 dpp

In the control animals of 90 dpp, gonocytes were no longer observed. Round and elongated spermatogonia, spermatocytes, and spermatids were seen in large numbers. At this age, unspecific alterations like germ cell hypoplasia, spermatocyte and spermatid

Table 2. Anatomical and histological parameters in the experimental groups.

Age (dpp)	Testicular weight/Body weight (gr)		Epithelial area (μm^2)		Histopathological index		Maturity index	
	Control	Estradiol	Control	Estradiol	Control	Estradiol	Control	Estradiol
60	0.0207±0.0007	0.0116±0.0005*	6746.78±113.00	3205.43±74.25*	9.29±0.29	12.38±0.39*	3±0	3±0
90	0.0575±0.0067	0.0187±0.0008*	23301.05±513.97	6997.07±135.21*	6.85±0.36	10.41±0.30*	6.83±0.15	2.96±0.01*
180	0.1326±0.0059	0.0299±0.0006*	42209.01±675.46	10527.02±223.78*	5.38±0.24	11.10±0.24*	9.50±0.16	3.35±0.04*

Mean (± S.E.M.) The data of each age group were compared with the control group, *$p<0.05$.

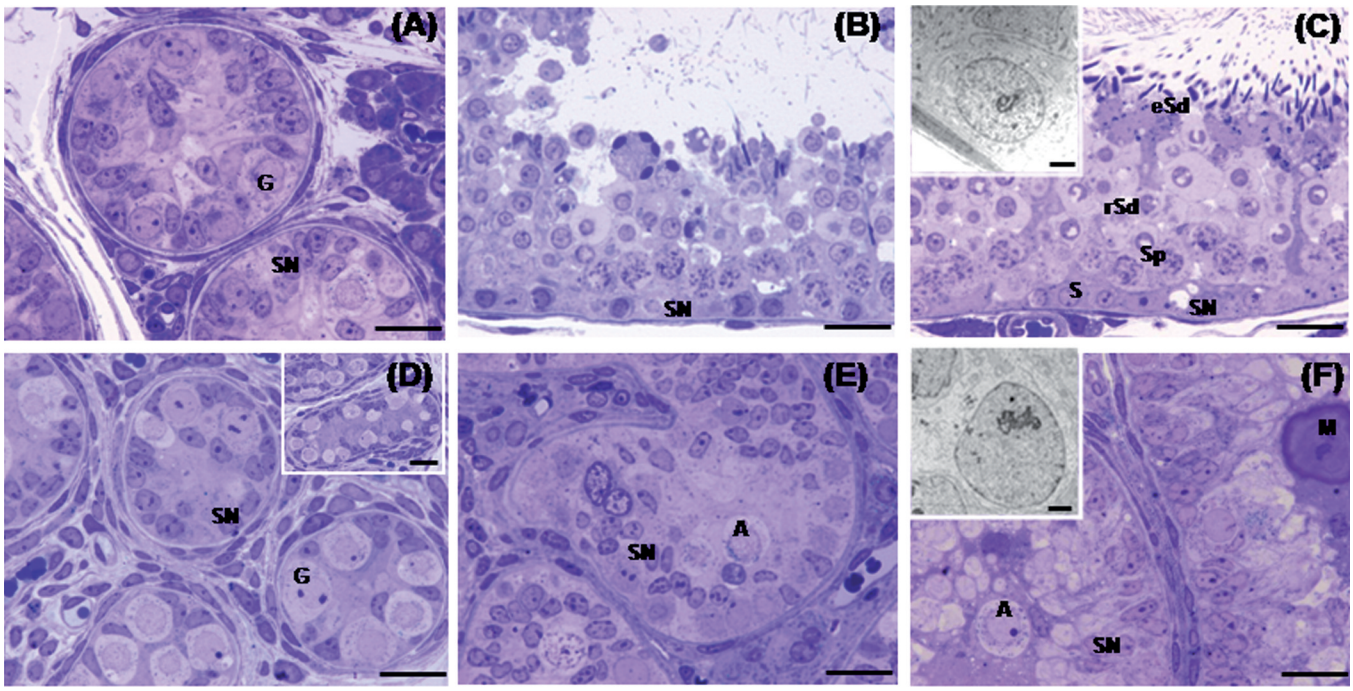

Fig. 1. Testicular images of the rabbit. The upper row corresponds to control animals and the lower row to estradiol group of animals. **A and D** 60 dpp; **B and E** 90 dpp; and **C and F** 180 dpp. The insets show the electron microscopy observations with spermatogonia in contact with the basal lamina (control) and gonocytes with ultrastructural characteristics typical of this type of cell - round and big cells, cytoplasmic low electrodensity, prominent nucleolus and few organelles (estradiol). G: gonocytes; SN: Sertoli cell nucleus; A: atypical cells; S: spermatogonia; Sp: spermatocytes; rSd: round spermatid; eSd: elongated spermatid; M: microlitiasis. Scale bars: 20 μm; insertion D, 60 μm; insertion C, F, 2 μm.

degenerations, germ cell vacuolization, and formation of multinucleated spermatid were observed (Fig. 11). The maturity and histopathological index, epithelial area, and the number of gonocytes and spermatogonia are shown in Table 2, Fig. 2.

The immunoreactivity to POU5F1 and c-kit proteins was not evident (Table 3, Figs. 3, 4) while Ki-67 was observed in cells as spermatogonia and spermatocytes in proliferations (Table 3, Fig. 5).

In the rabbits of the group exposed to estradiol, seminiferous epithelium presented atrophy which was characterized by detention in spermatogenesis, thickened basal lamina and folds in some zones (Fig. 1). A higher number of gonocytes and a lesser number of spermatogonia was observed when compared with the control group ($p<0.05$, Fig. 2). The maturity index and epithelial area were significantly less while the histopathological index increased significantly ($p<0.05$, Fig. 2) when compared with the control group.

The number of immunoreactive cells to POU5F1 and c-kit was significantly higher in comparison with the control group (Table 3, Figs. 3, 4), although, without significant difference in the number of cells in proliferation characterized by protein Ki-67 ($p>0.05$, Table 3, Fig. 5). The results of POU5F1 and c-kit were confirmed by RT-PCR (Fig. 6).

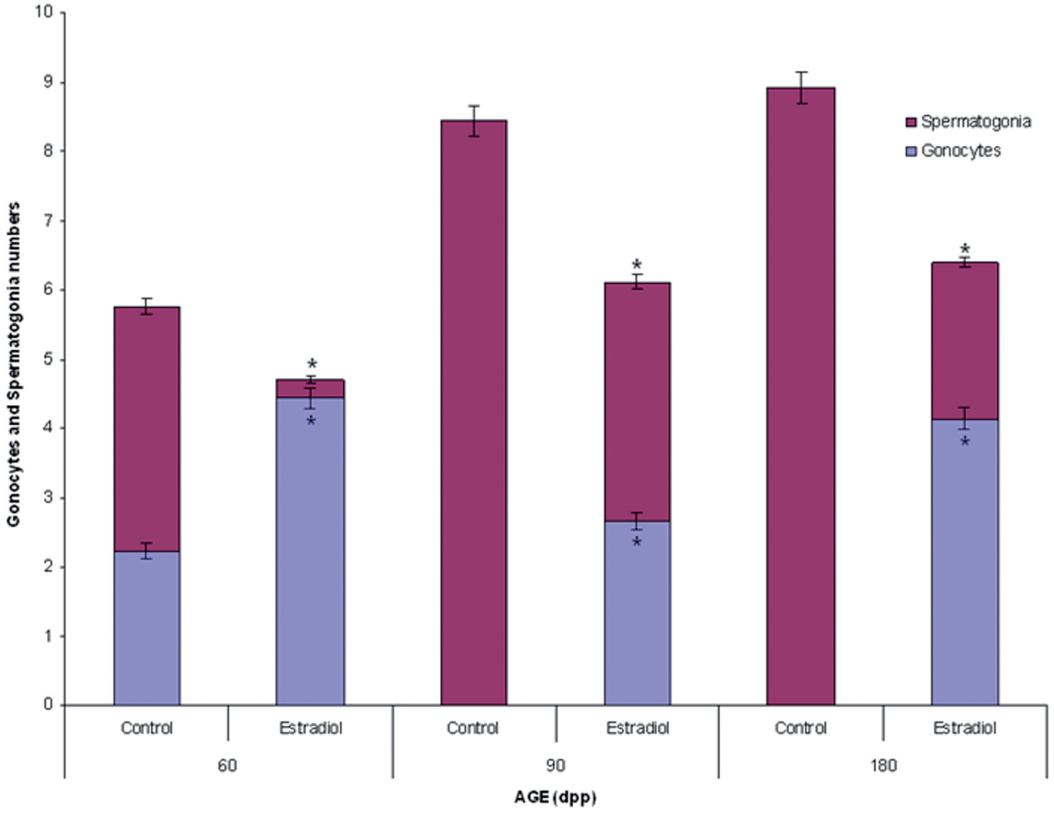

Fig. 2. Graphical demonstration of the number of gonocytes and spermatogonia per transverse section of seminiferous tubule. *p<0.05 vs control group of the same age.

Table 3. Immunohistochemical index for the different proteins studied/10 000 μm^2.

Age (dpp)	POU5F1		c-kit		Ki-67	
	Control	Estradiol	Control	Estradiol	Control	Estradiol
60	1.78±0.05	9.69±0.32*	2.18±0.09	9.77±0.18*	2.77±0.15	5.61±0.16*
90	0±0	1.93±0.07*	0±0	1.48±0.07*	9.85±0.36	10.15±0.19
180	0±0	9.58±0.73*	0±0	2.41±0.0872*	10.41±0.21	2.76±0.14*

Mean (± S.E.M.) The data of each age group were compared with the control group, *p<0.05.

Animals of 180 dpp

At 180 dpp, the seminiferous tubules of the control group showed a cytoarchitecture typical of the species with complete spermatogenesis and some pathologic signs as described in animals of 90 dpp. Electron microscopy observations revealed spermatogonia in contact with the basal lamina. The number of spermatogonia, maturity and histopathological index, and epithelial area are shown in Table 2, Figs. 1, 2. Immunoreactivity to POU5F1 was not observed (Table 3, Fig. 3). Immunoreactivity for c-kit in the achrosomes of the round spermatids was scarce, and Ki-67 was observed in cells like spermatogonia and spermatocytes in proliferation (Table 3, Figs. 4, 5).

In the tissues of estradiol exposed group at 180 dpp, detention of spermatogenesis, Sertoli cells in central position, thickened basal lamina, cellular disorganization, germ cell hypoplasia, and atypical cells were observed (Fig. 1) in homogeneous form in all parts of the testis. At electron microscopy level, it was confirmed that the atypical cells were persistent gonocytes with ultrastructural characteristics typical of this type of cell - round and big cells, cytoplasmic low electrodensity, prominent nucleolus, few organelles, and mitochondria surrounding the nucleus (Fig. 1). A significant reduction of the seminiferous epithelial area, maturity index and greater damage as reflected by histopathological index were observed in comparison with the control group (Table 2, Fig. 1). Moreover, a lesser number of spermatogonia and a significant increase in the number of gonocytes in the estradiol group were found ($p<0.05$, Figs. 1, 3).

The number of cells positive to POU5F1 and c-kit was significantly higher ($p<0.05$) with respect to the control group (Table 3, Figs. 3, 4). The results of POU5F1 and c-kit were confirmed by RT-PCR (Fig. 6). Ki-67 was present in the persistent gonocytes and spermatogonia (Table 3, Fig. 5).

Discussion

The rabbit is the smallest and most economic animal model for the study of various reproductive parameters that could be extrapolated to human beings (Foote and Carney, 2000), and in which reproductive parameters like ultrasound and testicular biopsy without spermatogenic function damage (Paufler and Foote, 1969) as well as semen collection by ejaculate (Seed et al., 1996) can be done. The human and the rabbit share a period of "childhood" that is well defined and which does not occur in rodents where the neonatal period overlaps with the period of infancy (Plant, 2006). Also, the seminiferous epithelium of the rabbit presents unspecific histological alterations like in human testis (Morton, 1988; Vigueras et al., 2013).

In this model, the administration of estradiol is similar to the exposure to endocrine disruptors during

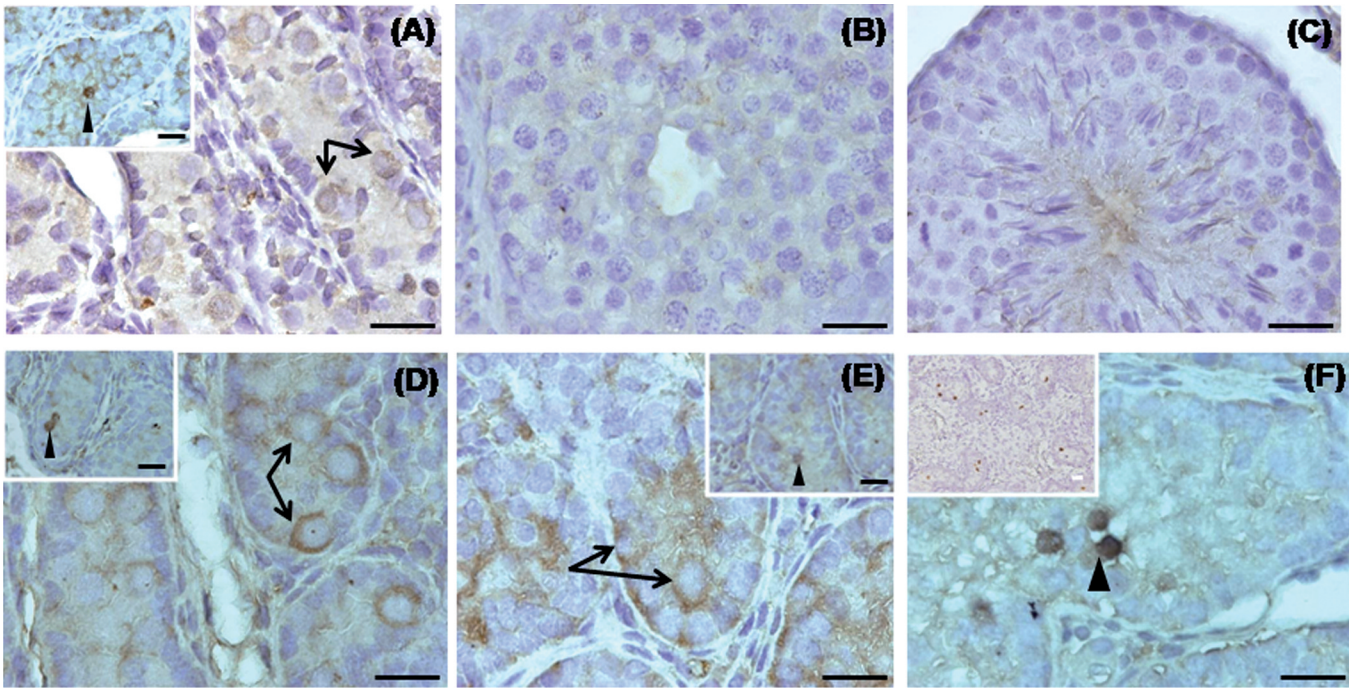

Fig. 3. Testicular immunohistochemical images of rabbits positive for cytoplasmic POU5F1 (arrow) and nucleic (arrow head). The upper row corresponds to control animals and the lower row to estradiol group. **A and D** 60 dpp; **B and E** 90 dpp; **C and F** 180 dpp. Bar scale: 20μm, insertion bar scale: 60 μm.

gestational and neonatal periods proposed as responsible for the increase in the incidence of TDS. There is little information on exposure to endocrine chemical disruptors during intrauterine or neonatal development in humans and their long-term effects (in adult life) since cause and effect are separated by a long time (Albert and Jégou, 2014). A lot of enviromental chemicals, including some pharmacological compounds, mimic the action of reproductive hormones, and in this way have the capacity to alter the neuroendocrine system or directly act on gonads. This can include an interplay between genetic-epigenetic and (micro-) environmental parameters referred to as "genviroment" (Looijenga et al., 2013).

The estrogens or compounds with estrogenic activity have been implicated as causal agents for the development of TDS (Sharpe and Skakkebaek, 1993; Toppari et al., 1996). Moreover, it is known that GPR30 is a 7-transmembrane G protein-coupled estrogen receptor that functions alongside traditional estrogen receptor to regulate cellular responses to 17 β-estradiol and enviromental estrogen and the presence in GCNIS has been reported (Franco et al., 2011).

GCNIS is the precursor of histological lesions in testicular cancer and has cryptorchidism as the risk factor, exactly as seen in our rabbits exposed to estradiol. Patients with cryptorchidism have a higher relative risk of 3.7 to 7.5 of developing TC than the normal population (Thorup et al., 2010). Fetal or neonatal gonocyte has been proposed as responsible for the development of GCNIS and it was suggested that this type of cell remains pluripotent without differentiating to spermatogonia in patients with cryptorchidism and possibly expresses proteins that confer to it this condition (Skakkebaek et al., 1987; Jorgensen et al., 1995; Rajpert-De Meyts et al., 2003; Looijenga et al., 2003; Honecker et al., 2004; Hoei-Hansen et al., 2005a; Sonne et al., 2009; Hutson et al., 2013). In addition, there was another proposal that these gonocytes proliferate in puberty after remaining quiescent during infancy, and later, progress to an invasive sickness under the influence of gonadotropins and/or testicular steroids (Skakkebaek et al., 1987).

In this model of rabbits, cellular proliferation at 60 dpp was favored by the administration of estradiol just as was reported by Moe-behrens et al., (2003) and Vigueras et al., (2006). On withdrawing estradiol, the number of gonocytes fell by more than 60%. At 90 dpp, the histological alterations such as delayed germ cell maturation, decreased germ cell number, lack of gonocytic differentiation, failure in the appearance of primary spermatocytes, and hypoplasia of the seminiferous tubules coincided with the alterations reported in the literature for patients with cryptorchidism from 6 to 12 months of age (Khatwa and Menon, 2000). At 180 dpp, the development of testicular hypoplasia occurred exactly as was reported in other species with administration of the same estradiol (Howdeshell et al., 2008) and this favored the persistence of the gonocytes without differentiating to spermatogonia, a fact that we

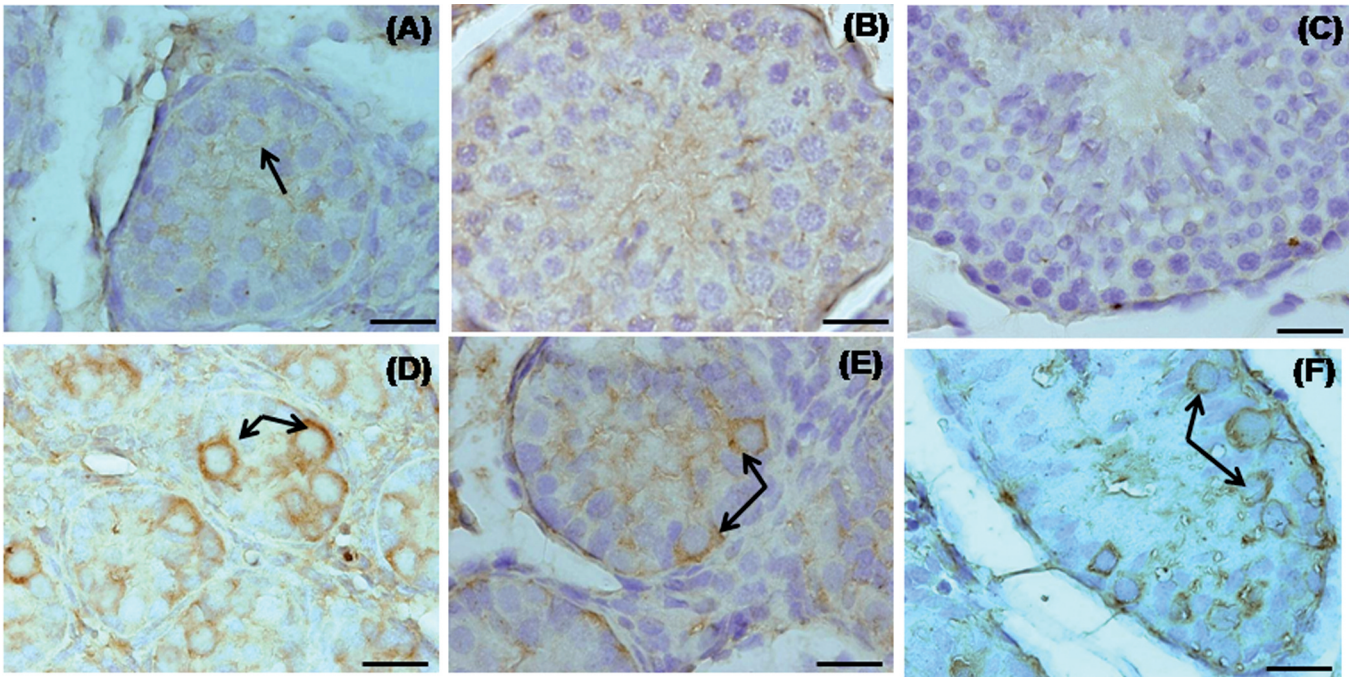

Fig. 4. Testicular immunohistochemical images of rabbits positive for c-kit (arrow). The upper row corresponds to control animals and the lower row to estradiol group. **A and D** 60 dpp; **B and E** 90 dpp; **C and F** 180 dpp. Bar scale: 20μm.

confirmed at the level of electron microscopy. The histological alterations such as atypical cells, thickened basal lamina, detention of spermatogenesis and movement of the Sertoli cells, coincided with Montironi (2002) for the diagnosis of GCNIS.

Recently, the prevalence of multifocality testicular cancer and germ cell neoplasia *in situ* (GCNIS) has been demonstrated. Both increased when the index mass tumor diameter was ≥1.1 cm. When organ-preserving tumor surgeries are carried out, it is necessary to consider the presence of adjacent GCNIS (Favilla et al., 2015), since it is known that 50% of men with GCNIS are at risk of invasive tumors within 5 years (Hoei-Hansen et al., 2005b). In this animal model, GCNIS development occurred in homogeneous form in all parts of the testis, therefore multifocal study was not developed.

Previously, Veeramachaneni (2006), in Duch-belted rabbits with administration of estradiol, reported the presence of testicular atypical cells. The positivity to markers such as POU5F1 and c-kit, determined in our model with estradiol, are considered as diagnostic markers of GCNIS (Looijenga et al., 2003).

In healthy children, testicular regulation of POU5F1

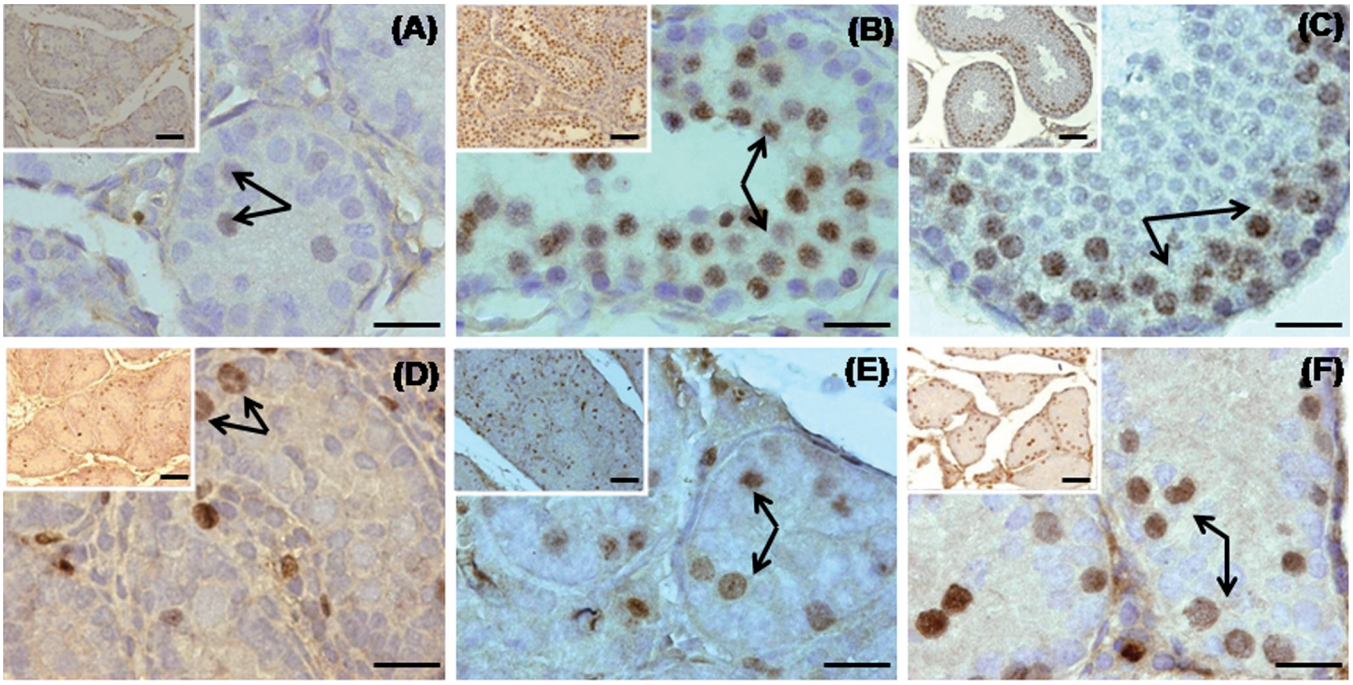

Fig. 5. Testicular immunohistochemical images of rabbits positive for Ki-67 as cellular proliferation marker (arrow). The upper row corresponds to control animals and the lower row to estradiol group. **A and D** 60 dpp; **B and E** 90 dpp; **C and F** 180 dpp. Scale bars: 20 μm; insertion, 60 μm.

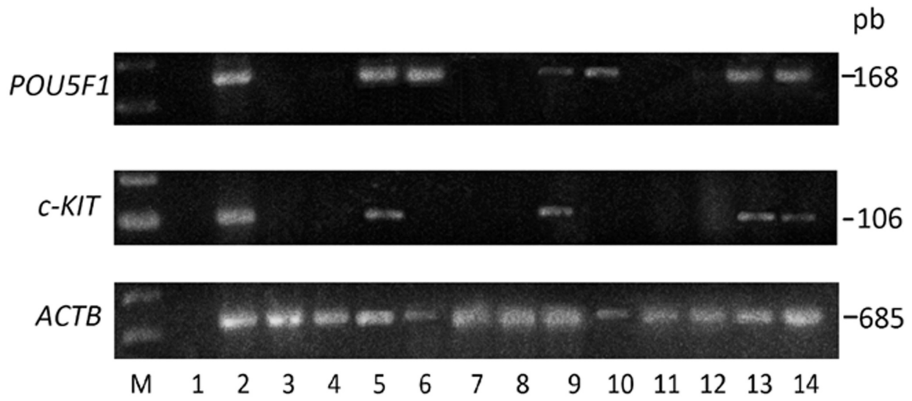

Fig. 6. POU5F1 and c-kit expressions in testicular tissue of control and estradiol rabbits of different ages. Line 1, negative control of the technique; line 2, positive control of a patient with POU5F1. Lines 3 and 4, control group; lines 5 and 6, estradiol group of 60 dpp. Lines 7 and 8, control group; lines 9 and 10 estradiol group of 90 dpp. Lines 11 and 12, control group; lines 13 and 14 estradiol group of 180 dpp. Molecular weight marker (M); base pairs (bp) and beta-actin (ACTB).

and c-kit has downward tendency (Rajpert-De Meyts et al., 2004). Our group reported that in patients with cryptorchidism, 5.4% presented germ cells that persisted in their expression of POU5F1 and c-kit proteins from early infancy until puberty (Vigueras et al., 2015). The immunoreactivity to these proteins in our control and estradiol exposed animals showed the same pattern as that observed in healthy and cryptorchid males. This demonstrates that with the administration of estradiol, the rabbit develops histological alterations and positivity to GCNIS specific markers (Jones et al., 2004; Cheng et al., 2007). POU5F1 is a member of the POU transcription factor family. It is expressed in embryonic stem and primordial cells of human and rat (Okamoto et al., 1990; Rosner et al., 1990). It is proposed that POU5F1 regulates cellular pluripotent capacity. This transcription factor is one of the key regulators of pluripotentiality (Pesce and Scholer, 2000). POU5F1 is present in CIS, gonadoblastoma, seminoma, germinoma, dysgerminoma, and embryonal carcinoma related with its pluripotent capacity (Skakkebaek et al., 1982; Palumbo et al., 2002).

It has been observed that mice embryonic stem cells exposed to estradiol continued with the transcription of POU5F1 gene, thereby favoring the undifferentiated state (Jung et al., 2010).

In our animals of 60 dpp, control and estradiol exposed, as well as those of 90 dpp in the estradiol group, POU5F1 protein was located in the cytoplasm and nucleus of the gonocytes. To date, three isoforms of POU5F1 have been documented (Wang et al., 2009; Wang and Dai, 2010) due to alternative splicing leading to the generation of the types: POU5F1A, B, and B1 (Takeda et al., 1992; Atlasi et al., 2008). It was suggested that POU5F1A, located in the nucleus as transcription factor, is responsible for maintaining the pluripotentiality properties of stem cells, while POU5F1B, located in the cytoplasm, is associated with stress responses (Wang et al., 2009). We consider that in our animals, POU5F1A was located in the nucleus of the gonocytes in order to maintain the pluripotentiality, while POU5F1B, located in the cytoplasm, permitted the survival of the gonocytes beyond their period of differentiation to spermatogonia. This is supported by the fact that in the control group of rabbits, the cells with cytoplasmic immunoreactivity degenerated around 90 dpp, but in the rabbits with testes that suffered caloric stress, generated by inguinal cryptorchidism originated by estradiol exposition, the gonocytes survived. This coincides with what was reported by Wang et al. (2009), who mentioned that in conditions of stress by heat, *Hela* cells are maintained living by POU5F1B and so inhibit their apoptosis. The expression of POU5F1 in the gonocytes and its persistence until 180 dpp in the estradiol group of our rabbits supports the theory of early origin of GCNIS from the undifferentiated persistent pluripotent gonocytes (Skakkebaek et al., 1987; Jorgensen et al., 1993; Rajpert-De Meyts and Skakkebaek, 1994; Rajpert-De Meyts et al., 1996; Jorgensen et al., 1997).

c-kit protein acts as a signal for survival, migration, and differentiation of early germ cells (Pesce et al., 1993; Runyan et al., 2006; Farini et al., 2007). c-kit is considered an anti-apoptotic factor and its over-expression could lead to survival of cells that remained undifferentiated and pluripotent (Bashamboo et al., 2006).

The interaction of SCF/c-kit increases the proliferation and suppresses the apoptosis of primordial germ cells (Pesce et al., 1993; Dolci et al., 2001; Tu et al., 2007). *In vitro* gonocytes, with the addition of SCF, were able to survive and proliferate, suggesting that this factor could have a role to play in mediating human gonocytic programmed cell death in developing testes (Helal et al., 2002).

Stoop et al. (2008) proposed that the prolonged expression of c-kit could contribute to neoplastic development which permits the survival of pluripotential cells. The expression of this protein was described, for the first time, in a tumor of germ cells by Strohmeyer et al. (1991) and in GCNIS by Rajpert De Meyts and Skakkebaek (1994).

c-kit gen has been described as a target of AP2γ transcription factor (Yasuda et al., 1993). Therefore, the prolonged expression of AP2γ and permanent activation of c-kit could have effects on malignant transformation by increasing the survival of immature germ cells and detention of germ cell differentiations.

In this model, we propose that the administered estradiol in the rabbits acted on different cell types as hypothesized below: 1) directly on Sertoli cells, known to present estradiol receptors (Fietz et al., 2014); 2) directly on gonocytes, which also have estradiol receptors, and possibly inducing a) lack of response to the factors produced by Sertoli cells for their differentiation, b) absence of down-regulation of their POU5F1 and c-kit proteins; 3) via Sertoli cells or gonocytes to impede the apoptosis of the gonocytes and to maintain them as undifferentiated and pluripotent.

On the other hand, it is also possible that cryptorchid-associated oxidative stress induces the development of neoplasia (Gupta et al., 2014). The arguments in favor of this are based on the fact that testis in abdominal position associated with high temperature is prone to GCNIS development (Ogunbiyi et al., 1996). Moreover, early orchidopexy reduces the risk of developing GCNIS (Pettersson et al., 2007; Walsh et al., 2007).

Veramachaneni (2006) in surgically developed cryptorchidism did not observe the presence of atypical cells like in estradiol exposed rabbits. This might be due to the fact that at the time of surgery (21 dpp), the testes are localized in the trajectory of testicular descent, since this process finishes around 50 dpp.

Our rabbit model developed GCNIS with the histological characteristics and presence of specific markers of this pathology. We demonstrate that a population of the gonocytes persisted without

morphological changes or differentiation to spermatogonia and so conserved their pluripotentiality. Therefore, we could conclude that the rabbit is a model that could be employed for the study of etiological and physiopathological mechanisms of GCNIS, as well as a providing the basis for future searches for non-invasive early diagnostic biomarkers of the disease which would serve as a guide for starting preventive treatment alternatives in order to reduce the risk of illness.

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