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Gonadal Differentiation During Embryonic and Fetal Development of Male New Zealand Rabbits (*Oryctolagus cuniculus*)

Diferenciación Gonadal Durante el Desarrollo Embrionario y Fetal de Conejos Machos Neozelandeses (*Oryctolagus cuniculus*)

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SUMMARY: The rabbit is considered an ideal animal model for studies that describe abnormalities in the testicles due to the similar morphogenetic mechanisms of sexual development and diseases commonly found in humans. The aim of this study was to determine the male sexual differentiation of the New Zealand rabbit (*Oryctolagus cuniculus*) through development. The gestational age was estimated and classified as 9, 12, 14, 16, 18, 20, 23 and 28 gestational days. The morphological and sexual determination were performed by histological analysis of the reproductive tract in the embryos and fetuses (9-28 days) as well as by immunohistochemistry-Desert hedgehog-Dhh– (testis-specific protein on Y chromosome– 16, 20, 23 days and adult rabbits). Gonads were observed from the 14th day in an undifferentiated stage and with homogeneous aspect. Sexual differentiation was observed from the 16th day with presence of cells forming gonadal cords and Dhh^{+} cells in the gonadal parenchyma. From the 18th gestational day testicular cords were observed, which evolved into organized seminiferous tubules. The formation of the efferent ducts and ductus deferens and epididymis was observed on the 20th and 23rd days, respectively. The differentiation of the external genitalia occurred from the 23rd days from the anogenital distance and was identified to identify the penile structures. In summary, the features of the sexual differentiation were determined by observation of the Dhh^{+} protein in embryos from the 16th day to adulthood, and the morphological particularities observed from the 18th gestational day, determined by differentiation of the external genitalia from the 23rd day.

KEY WORDS: Gonads; Embryonic development; Seminiferous tubules; Desert hedgehog; Sustentacular cells; Primordial germ cells.

INTRODUCTION

The *Oryctolagus cuniculus* species presents a sequence of genes more similar to human than other species such as rodents, for instance mouse and rat; therefore, they represent the third most used laboratory animal in experiments with mammals seeking to understand human diseases and in toxicological and chemical tests related to dermatology, embryology and reproductive biology (Püschel *et al.*, 2010).

The perinatal period is extensively studied in animal models to explain the development of congenital malformation events (Beaudoin *et al*., 2003; Booth *et al*., 2013). Moreover, the increased incidence of reproductive diseases in men, such as testicular cancer, poor semen quality and cryptorchidism may be correlated and characterized as testicular dysgenesis syndrome, caused by modifications in embryonic and gonadal development (Skakkebaek *et al*., 2003).

In mammals, gonadal differentiation begins during the intrauterine period. First, the genital ridge appears from the mesonephros, which are colonized by germ and somatic

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cells, and then differentiated in spermatozoa (sperm) as cells expressing steroids (interstitial cells, IC) and cells supporting germ cells (sustentacular cells, SC) (Daniel-Carlier *et al*., 2013). The interaction between these types of cells is fundamental from the fetal period to puberty to guarantee gonadal homeostasis; otherwise modification of the spermatogenesis may occur and increase the predisposition to neoplasms (Banco *et al*., 2016).

The molecular events that occur during such period are widely studied in mice and rats. However, those species do not develop testicular diseases analogous to those found in human, for instance neoplasms (Banco *et al*., 2016), and their embryonic period is extremely short and there are some differences considering the duration of other embryonic events. Therefore, the studies are restricted to explaining some modifications in gonadal development, identified in other species, such as human and dogs (Brennan & Capel, 2004; Grieco *et al*., 2008).

By contrast, the rabbit is considered a more suitable animal model for studies examining testicle abnormalities (Polisca *et al*., 2010) due to its similarities with diseases commonly found in humans such as testicular neoplasms and seminomas (Banco *et al*., 2012; Suzuki *et al*., 2011). However, studies with rabbits regarding their reproductive gonadal development are limited.

Therefore, the aim of this study was to determine male sexual differentiation using morphological and tissue analyses and immunohistochemistry of gonads of New Zealand White rabbits through embryonic and fetal development.

MATERIAL AND METHOD

Animals. The embryos and fetuses of New Zealand rabbits (n=28) were obtained from the Cuniculture Department of the University of São Paulo (USP)/ Pirassununga college and the sample approaches were performed at the Histology Laboratory of USP, São Paulo (USP). This present study was approved by the Research Ethics Committee of the University of São Paulo/ Pirassununga (protocol number 13.11910.74.9).

Ages and morphological description. The age was estimated considering the external features, as well as the crown-rump length of embryos and fetuses according to Evans & Sack (1973); a measurement from the first cervical vertebra to the last caudal vertebra was made. Finally, the samples were organized in 9, 12, 14, 16, 18, 20, 23 and 28 gestational days (Table I).

Table I. Ages of New Zealand rabbits according to crown-rump length of embryos and fetuses (Evans & Sack).

Ages (days)	Mean of length (cm)
9	No detected
12	0.5
14	1.1
16	1.5
18	2.1
20	3.9
23	4.3
28	6.7

All morphological features were analyzed using a stereo microscope (Stemi SV6, Zeiss) and documented (Sony Cyber-shot). The samples were dissected between 20 and 28 days to observe the inner structures of the male reproductive tract.

Histological analysis. Embryos between 9 and 16 days and the reproductive tracts which were dissected (animals between 18 and 28 days) were fixed with 4 % paraformaldehyde for 48 hours, gradually dehydrated using ethanol (70 % - 100 %, Synth), incubated with xylene (Dynamics) and embedded in paraffin wax (ERVPLAST, EasyPath). 5 µm sections were cut using a Leica manual microtome (RM2165, Germany) and stained with hematoxylin-eosin. Finally, the sections were documented using a Nikon 80i light microscope.

Immunohistochemical analysis . The immunohistochemical analysis were performed using the Cell & Tissue Staining HRP-DAB System kit (R & D Systems, Goat Kit, CTS008) for Desert hedgehog (Dhh), a protein found in the membrane of germ cells, in the testicles of adult rabbits and those with 16, 20 and 23 days of embryonic/fetal development. Sections were dewaxed with xylene and hydrated in decreasing concentrations of ethanol, followed by citrate buffer 0.1 M pH 6.0 and heated with a vaporizer for 30 minutes to antigen retrieval. After blocking, endogenous peroxidase and nonspecific proteins, incubation was performed with primary antibodies Ihh (1:100; C-15, sc-1196, Santa Cruz Biotechnology, polyclonal, goat) and Dhh (1:100; N-17, sc-33940, Santa Cruz Biotechnology, polyclonal, goat) in a humidity chamber at 37˚C for 90 minutes. The negative control was performed as described using Phosphate buffered saline (PBS 1x) instead of primary antibody.

RESULTS

The crown-rump length and the external features of embryos and fetuses of the New Zealand rabbits were obtained according to Evans & Sack for the determination of gestational age.

On the 9th day of embryonic development, the implantation site (Fig. 1A) was visible by dilatation in the uterine horn, containing the embryo surrounded by amnion. Microscopically, the embryos were observed with the three embryonic folding: the ectoderm, composed of cylindrical cells; the mesoderm, highlighting three regions: paraxial, intermediate and lateral mesoderm; and the endoderm. The intermediate mesoderm is responsible for the development of the urogenital system. Moreover, the neural tube and early organogenesis, e.g., heart development, were observed (Fig. 1B).

The mean embryo size on the 12th day of development was 0.5 cm, characterized by a dorsally convex curvature (C shape) and spiral torsion of the final part of the tail, presence of fore and hind limb buds, formation of the tail, evident heart and liver, and the presence of the pharyngeal arch, composed of the first pharyngeal arch (mandibular arch), second pharyngeal arch (hyoid arch) and third pharyngeal arch (Fig. 1C). In histological analysis, several organs, e.g., brain, tongue, esophagus, somites, heart, lung, liver, gut were observed; however, the gonads had not been formed yet (Fig. 1D). In this stage, most primordial germ cells (PGCs) were situated in the gut wall, and then they would migrate to the intestinal mesentery and to the gonadal ridge in formation.

On the 14th day of development, the embryos measured an average of 1.1 cm, showing defined and pigmented eyes, the presence of the buds of the hands and feet with grooves between the digits and pinna in formation as a ridge (Fig. 2A). The primordial gonad developed from an elongated region of the mesoderm, along the ventromedial portion of the mesonephros, establishing communication from the connective tissue and showing a homogenous granular appearance with several cell populations, called the undifferentiated gonad (Figs. 2B - 2E). The mesonephro were composed of mesonephric ducts and tubules, responsible for the formation of the glomeruli at the medial extremity; the metanephros, precursor of the permanent kidney, were also visualized in this stage (Fig. 2D).

On the 16th day of development, the embryos measured an average of 1.5 cm, characterized by head elevation with the presence of a ridge in the right angle, pinna partially surrounded by acoustic meatus, onset the formation of the eyelids, tactile hairs in the upper region of the lips and below the eyes, and the main feature of this stage was the herniation of the intestine (Fig. 2F). In the histological analysis, the gonad growth concomitant with mesonephros degeneration was observed (Figs. 2G - 2I), which is the onset of male sexual differentiation. Therefore, the gonads showed different cell populations composed of

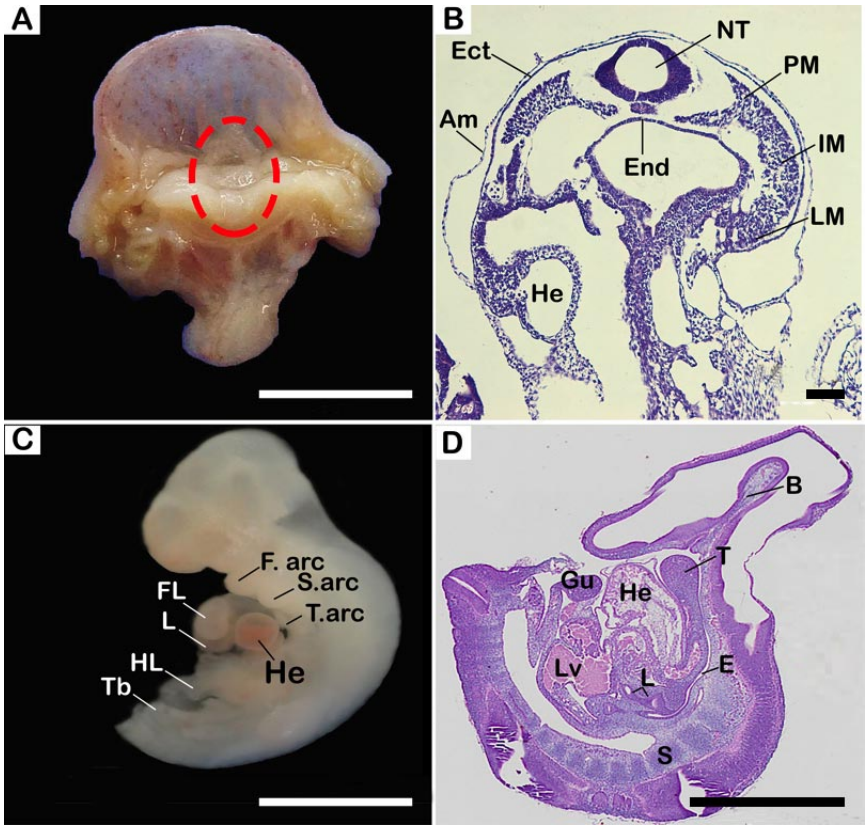

Fig. 1. Embryonic bud of New Zealand rabbit on the 9th day of development, highlighting the implantation site (red circle) (A). Embryo on the 9th day containing the three embryonic folding (ectoderm, mesoderm and endoderm), neural tube and heart (B). Embryo on the 12th day of development (C). Embryo on the 12th day, highlighting organogenesis; however, the gonads were not developed yet (D). Am: amnion; Ect: ectoderm; PM: paraxial mesoderm; IM: intermediate mesoderm; LM: lateral mesoderm; End: endoderm; NT: neural tube; He: heart; F. arc: first pharyngeal arch (mandibular); S. arc: second pharyngeal arch (hyoid), T. arc: third pharyngeal arch; FL: forelimb; L: lung; HL: hindlimb; Tb: tail bud; B: brain; T: tongue; E: esophagus; Lv: liver; Gu: gut. H-E staining. Scale bar (A, C): 1 cm; (B): 100 µm; (D): 1 mm.

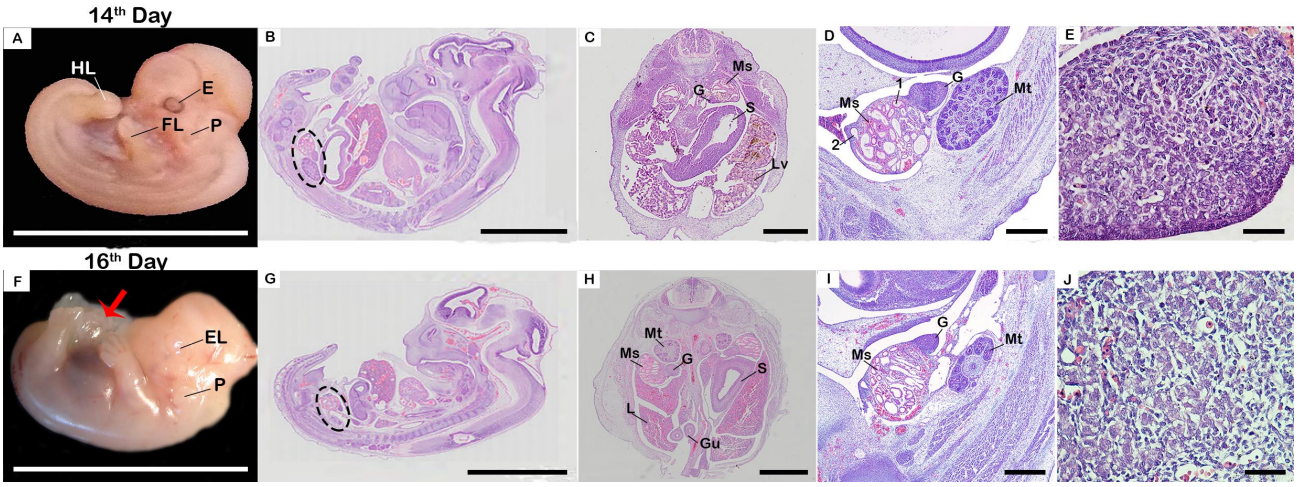

Fig. 2. Embryo of New Zealand rabbit on the 14th (A-E) and 16th (F-J) days of development, showing the morphology of the embryos and the position of the gonads in an undifferentiated stage and homogeneous aspect, along the ventromedial portion of the mesonephros. E: eyes; P: pinna; FL: forelimb; HL: hindlimb; Ms: mesonephros; G: undifferentiated gonad; S: stomach; Lv: liver; Mt: metanephros; Gu: gut; EL: eyelid. Red arrow: herniation of the intestine. 1: mesonephric tubules; 2: mesonephric ducts. H-E staining. Scale bar (A, F): 1 cm; (B, G): 2 mm; (H): 1 mm; (C, D, H, I): 500 µm; (E-J): 50 µm.

local mesenchymal cells, coelomic epithelial cells and mesonephric tubules cells in degeneration process; these cells invaded into gonadal parenchyma, forming irregular gonadal cords and incorporating the PGCs (Fig. 2J).

On the 18th day of development the embryos measured an average of 2.1 cm, highlighting the presence of eyelids partially covering the eyes and separated arranged digits (Fig. 3A). Microscopically, the mesonephro were observed at the degeneration stage, still connected to the gonad by the connective tissue (Fig. 3B), presenting initial organization of the cells of the testicular or medullary cords, which contained PGCs and sustentacular cells (SCs), isolated from the stroma by blood vessels and the basal lamina (Figs. 3C, 3D).

The 20th day of development, the embryos measured an average of 3.9 cm, with fusion of the eyelids, pinna covering more than half of the external acoustic meatus, presence of claws, and return of the gut into the abdominal cavity (Fig. 3E). The gonad was situated caudally to the metanephros and cranially to the mesonephro (Fig. 3F). In this stage, the external genitalia had not yet been sexually differentiated, although histological analysis showed the structure of the penis, spongy body surrounding the urethra and corpora cavernosa (Fig. 3G), as well as gonads containing seminiferous tubules organized and composed peripherally of SCs and several PGCs more centrally, being surrounded by contractile myoid cells brought about by the migration of mesonephric cells. In the epithelium, we observed the organization of mesenchymal cells underlying the coelomic epithelium, forming the tunica albuginea (Figs. 3H - 3J). The ductus deferens in the transverse section presented mucosa composed of a lumen with a stellate shape and surrounded by an epithelium composed of cylindrical pseudo stratified cells, basal membrane and a thick and organized muscle tunica (Fig. 3K). In the testicular mediastinum, we observed the formation of the efferent tubules formed by several canaliculi, which would then end in the head of the epididymis; however, this was not visualized in this stage (Fig. 3L).

On the 23rd day the fetuses measured an average of 4.3 cm (Fig. 4A) and showed sexual differentiation of the external genitalia (Fig. 4B). With respect to the topography of the internal sexual organs, the position of the gonads was observed immediately cauda to the metanephro and specifically the male gonads were slightly larger than the female gonads (Fig. 4C). Microscopically, observations were made of the internal structure of the penis (corpora cavernosa and spongy body) (Fig. 4D), mesonephro in an advanced stage of degeneration, containing tubules highly disorganized and mesonephric ducts in the process of differentiation in the extratesticular genital ducts (epididymis) (Fig. 4E). The testicular parenchyma presented several primitive seminiferous tubules, containing PGCs, SCs and myoid cells surrounding the tubules, beyond the interstitial cells; however, it was not possible to distinguish them (Fig. 4F). In this stage, the epididymis covered the cranial pole of the gonad; efferent ducts were observed coming out of the testicle, and composed of a lumen and surrounded by cylindrical pseudostratified cells (Fig. 4G).

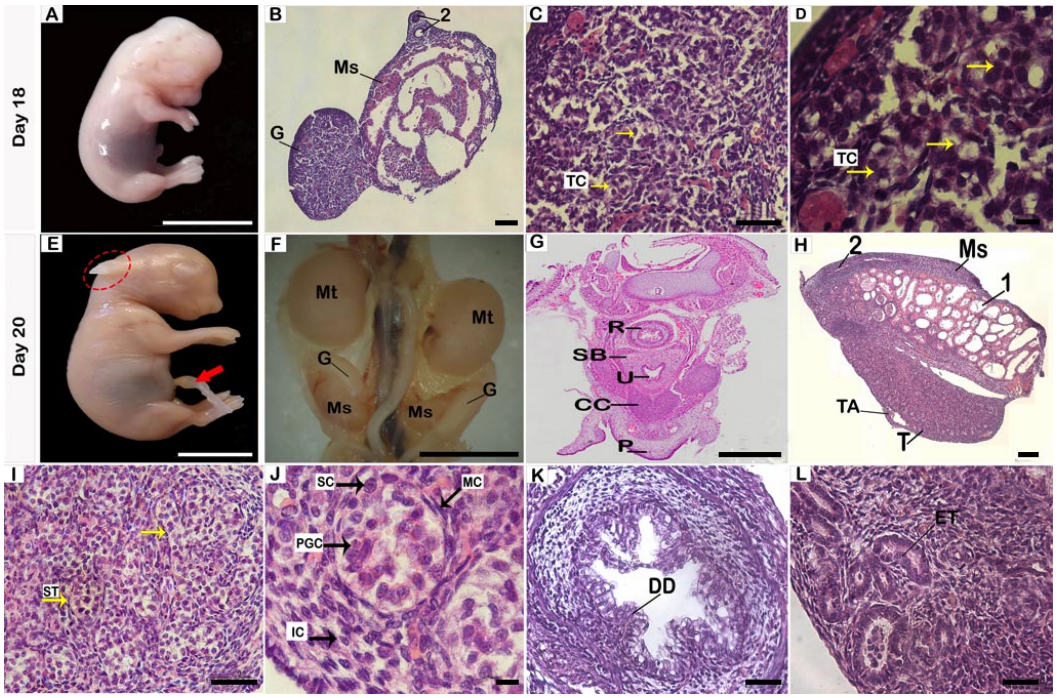

Fig. 3. Embryo of New Zealand rabbit on the 18th (A-D) and 20th (E-L) days of development. Differentiated gonads were observed cranially to the mesonephros and characterized by (D) testicular cord on the 18th days (I) and primitive seminiferous tubules on the 20th days. (DD) Ductus deferens and (ET) efferent tubules were visualized. Ms: mesonephros; G: gonad; Mt: metanephros; TC: testicular cord; R: rectum; SB: spongy body; U: urethra; CC: corpora cavernosa; P: prepuce; T: testicle; TA: tunica albuginea; ST: seminiferous tubules; SC: sustentacular cells; PGC: primordial germinative cells; IC: interstitial cells; DD: ductus deferens; ET: efferent tubules. Red arrow: umbilical cord; 1: mesonephric tubules; 2: mesonephric ducts. H-E staining. Scale bar (A, E, F): 1 cm; (G): 500 µm; (H): 200 µm; (B): 100 µm; (C, D, I, K, L): 50 µm; (D, J): 10 µm.

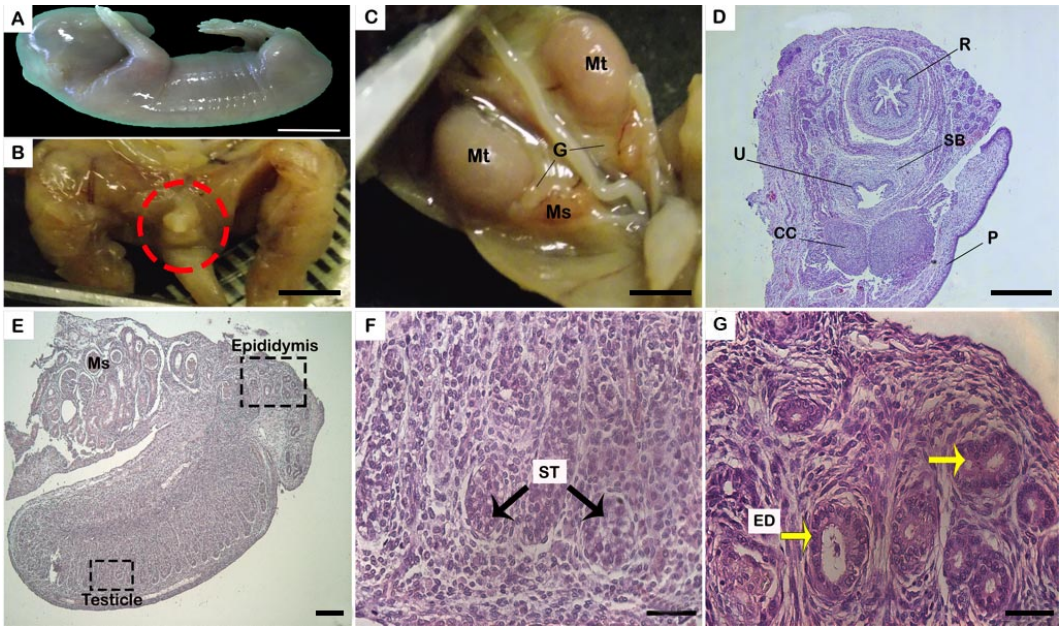

Fig. 4. New Zealand rabbit male fetus on the 23rd day of development (A), showing differentiation of external genitalia (B); the appearance of the male gonads slightly larger than the female gonads (C). Histology analysis showed penis structure (D), seminiferous tubules, epididymis and efferent ducts (E-G). Mt: metanephros; Ms: mesonephros; G: gonad; R: rectum; SB: spongy body; U: urethra; CC: corpora cavernosa; P: prepuce; ST: seminiferous tubules; ED: efferent ducts. H-E staining. Scale bar (A-C): 1 cm; (D): 500 µm; (E): 200 µm; (F, G): 50 µm.

At the end of fetus development on the 28th day, the fetuses measured an average of 6.7 cm and the same features described at the previous age, highlighting the presence of hairs all over the body (Fig. 5A). In the topography of the internal sexual organs, the gonads showed a slightly more ovoid shape, with a larger volume and situated more caudally to the kidney, close to pelvic region. Laterally to the testicle, ductus deferens were observed connected to the gonads by an extension of the fascia layer, which terminates in the epididymis (situated cranially to the testicle; Figs. 5B - 5D). The seminiferous tubules presented communication with canaliculi of the testicular network, and internally a more defined lumen was observed filled centrally by the PGCs, myoid cells and SCs situated close to the basal membrane and with an evident nucleolus. A considerable volume of mesenchymal tissue was noted in the parenchyma, composed of the precursors of the interstitial cells, responsible for hormoneproduction, influencing the differentiation of the system of genital ducts (Figs. 5E, 5F). At this final stage, we observed the growth of the epididymis, characterized by more defined efferent ducts in the head of the epididymis, originating from testicle and epididymal ducts, in addition to the body and tail of the epididymis (Figs. 5G - 5I).

With regard to external genitalia of the fetuses on the 28th day, we observed the perineum of the male rabbit

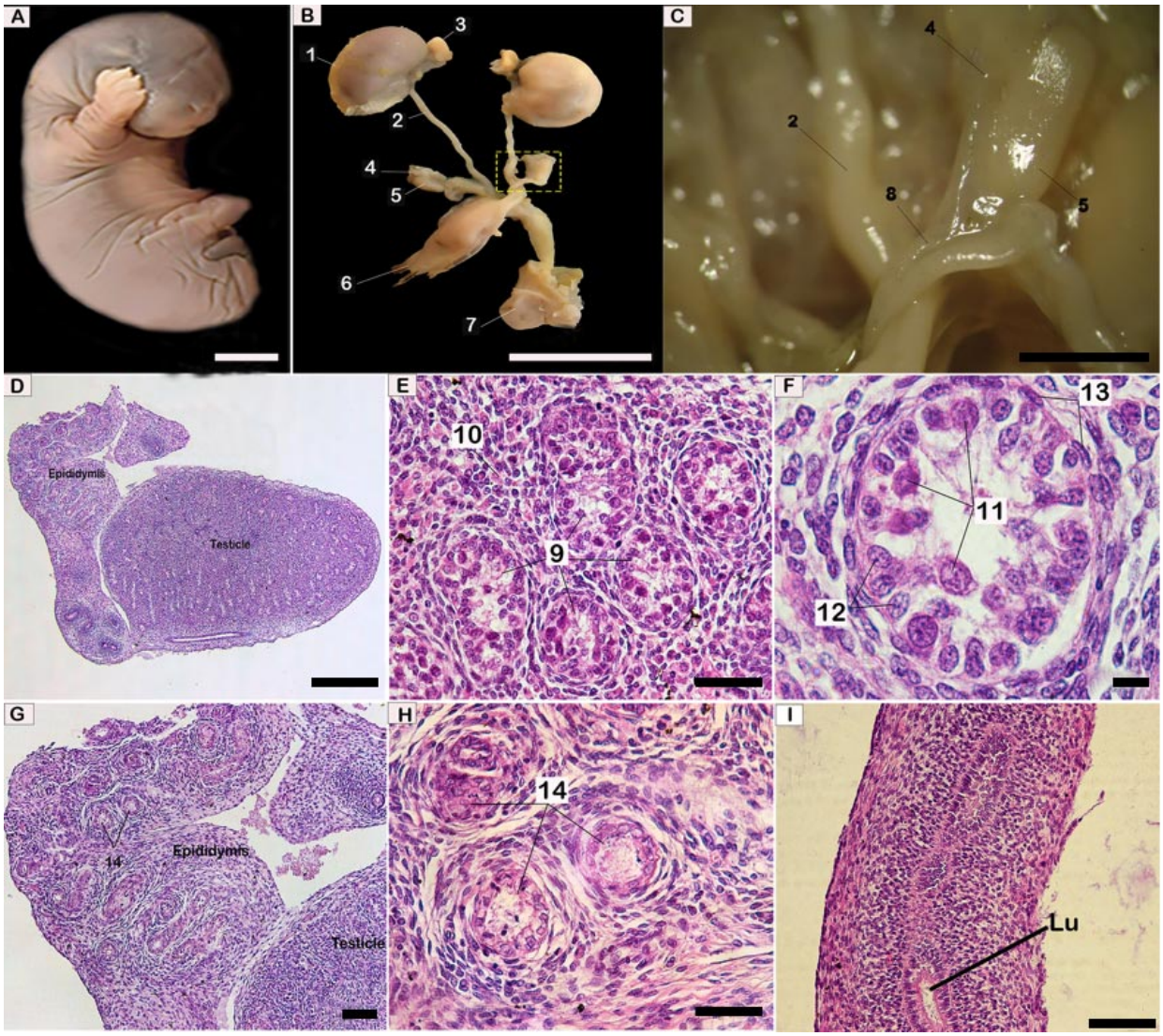

Fig. 5. New Zealand rabbit male fetus on the 28th day of development, highlighting the maturation of the male urogenital tract (A-C). Well-defined preview of the cell components of the seminiferous tubules (D-F) and epididymis (G-I). 1: kidney; 2: ureter; 3: adrenal gland; 4: epididymis; 5: testicle; 6: bladder; 7: genital tubercle; 8: gubernaculum; 9: seminiferous tubules; 10: testicular interstice; 11: primordial germinative cells; 12: sustentacular cells; 13: myoid cells; 14: efferent ducts; Lu: lumen. H-E staining. Scale bar (A-C): 1 cm; (D): 500 µm; (E, H): 50 µm; (F): 10 µm; (G, I): 100 µm.

was longer than in the female (Fig. 6A). The penis was composed of corpora cavernosa and spongy body (Figs. 6B, 6C). The corpora cavernosa was situated in the dorsal region, containing the internal deep artery and more abundant vascular spaces (Fig. 6D). The spongy body was characterized by several vascular spaces, surrounding the urethra, and delimited by a layer of connective tissue, composed of elastic fibers, collagen fibers and isolated mucosa glands (Fig. 6E). The bulbourethral glands were situated in the portion of the membranous urethra, presenting alveolar tubules made up of a simple cubic epithelium secreting mucus and surrounded by a capsule of connective tissue (Fig. 6F). Finally, the penile urethra was identified within the spongy body, forming many folds in which the urethral glands ended, and it was composed of a pseudostratified epithelium or cylindrical stratified epithelium and a stellate lumen, surrounded by the lamina propria of the mucosa (Figs. 6G, 6H).

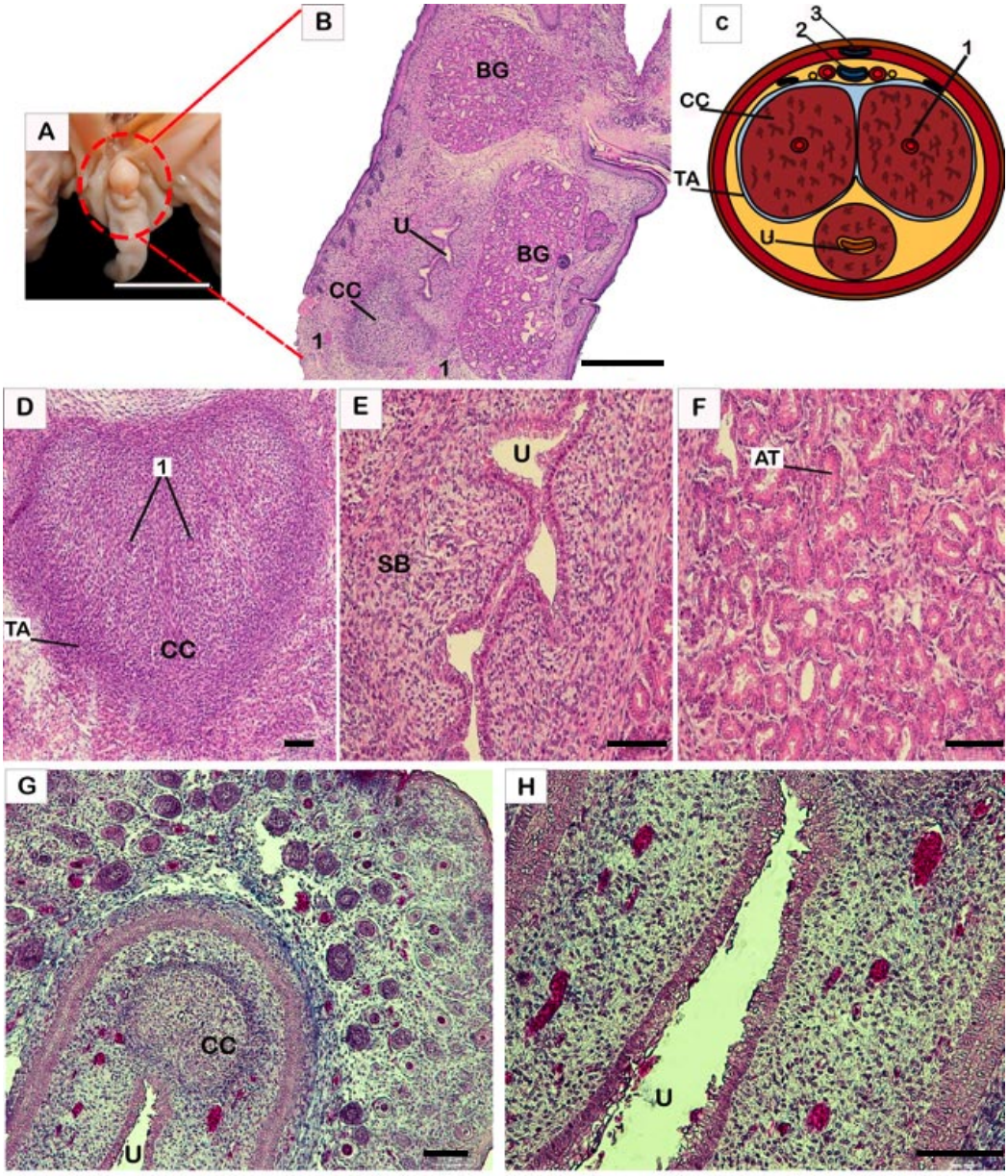

Fig. 6. External genitalia of New Zealand rabbit male fetus on the 28th day of development (A), highlighting the structure of the penis and bulbourethral gland (B-H). Schematic design of the transverse section of the penis (C). BG: bulbourethral gland; U: spongy urethra, surrounded by the spongy body; CC: corpora cavernosa; TA: tunica albuginea; SB: spongy body; AT: alveolar tubules. 1: deep artery (corpora cavernosa); 2: deep dorsal vein; 3: superficial dorsal vein. H-E and Masson's Trichrome staining. Scale bar (A): 1 cm; (B): 500 μm; (D-H): 100 μm.

The immunohistochemical analysis showed the presence of Dhh$^+$ germ cells in the gonadal parenchyma in rabbits on the 16th day of embryonic development (Fig. 7A). From the 20th and 23rd days of gestation, Dhh$^+$ cells were observed in the epithelial network- a precursor of the seminiferous cords (Fig. 7B)- within the seminiferous tubules (pre-sustentacular cells and PGCs) and in the interstitium (pre-interstitial cells) (Figure 7C). In the adult animal, Dhh$^+$ cells were observed in sustentacular cells and in spermatogenic lineage cells situated peripherally in the seminiferous tubules and more centrally toward the lumen, respectively (Figs. 7D, 7E).

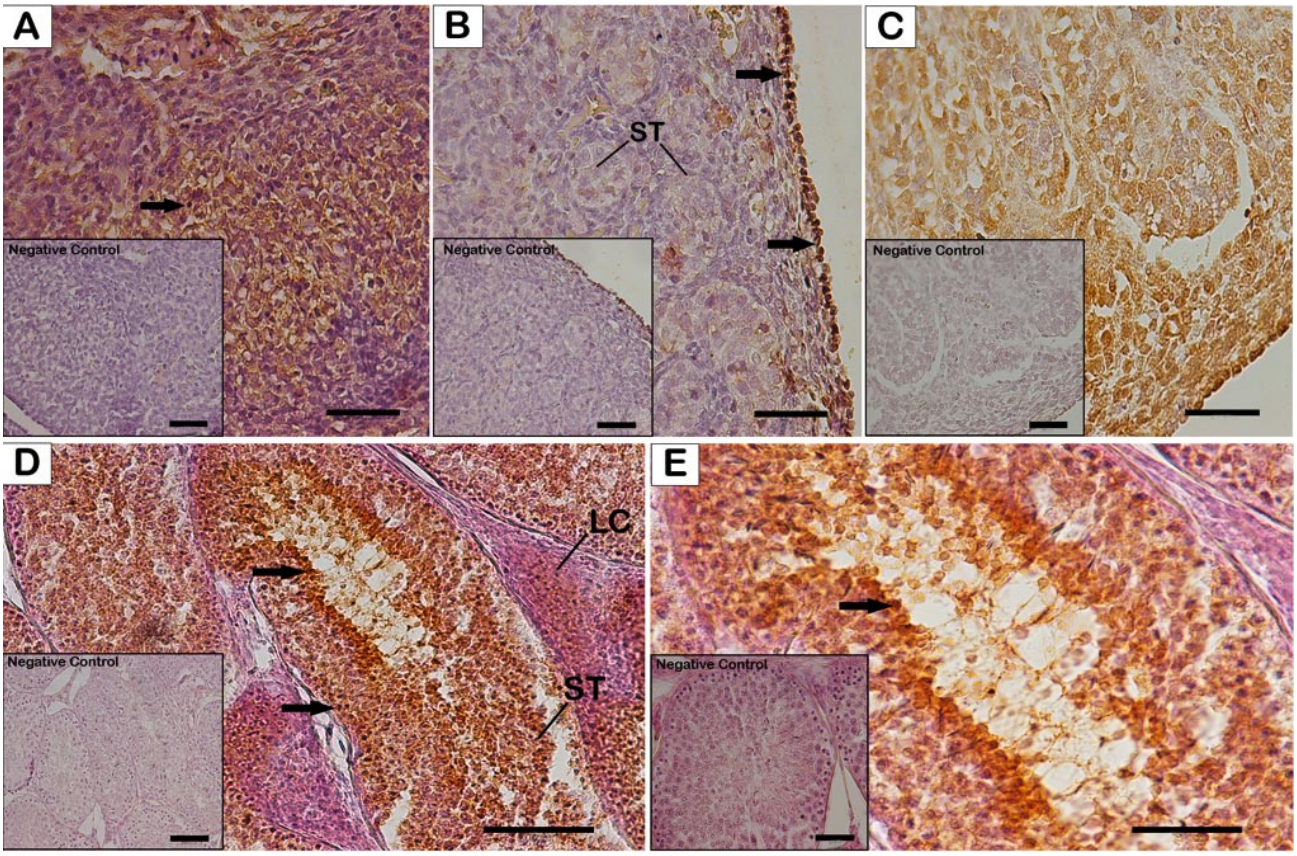

Fig. 7. Immunohistochemical analysis of Dhh$^+$ (Desert hedgehog positive) germ cells in gonads of the New Zealand rabbit, characterizing the onset of male sexual differentiation. (A) Dhh+ germ cells in the histologically undifferentiated gonadal parenchyma on the 16th day of embryonic development. (B) Dhh$^+$ cells in germinal epithelium of the differentiated gonad on 20$^+$ day. (C) Dhh$^+$ cells within the seminiferous tubules, interstitium and epithelium on 23rd day of fetal development. (D, E) Dhh$^+$ cells in sustentacular and spermatogenic lineage cells in the adult animal. ST: seminiferous tubule; Black arrow: Dhh$^+$ cells. Scale bar (A, B, C, E): 50 μm; (D): 100 μm.

DISCUSSION

The rabbit is an ideal animal model due to its small size low maintenance cost and easy handling. It is used for different kinds of research, e.g. metabolic diseases and neoplasm (Bosze & Houdebine, 2010), embryonic development (Beaudoin *et al.*), toxicology of the male reproductive tract in humans (Attardi *et al.*, 2011), cardiovascular diseases (Guo *et al.*, 2012), environmental toxicology tests (Kawamura *et al.*, 2013) and congenital malformations (Booth *et al.*).

In reproduction, morphogenetic mechanisms that occur during the development and differentiation of the male gonad in the rabbit are more similar to those that occur in other mammals, including humans, than in mice (Díaz-Hernández *et al.*, 2008). In addition, this species is widely used in pharmacological studies as models for carcinoma in situ of testicles (Rao Veeramachaneni, 2006).

The present study reported the sexual differentiation of New Zealand rabbits from 9 to 28 days of embryonic/ fetal development. Sexual differentiation in mammals starts during the intrauterine period. The PGCs appear in the rabbit

on the 9th day of embryonic development, whereas in the mouse it occurs on the 7th day, and then migrates through the dorsal mesentery to the gonadal ridge, where successive mitosis occurs (Daniel-Carlier *et al*.; Wilhelm *et al*., 2007).

The gonads became evident from 14th day of development, from the thickening of the coelomic epithelium, presenting morphologically in an undifferentiated stage and connected to the mesonephros, as reported for chinchillas (Díaz-Hernández *et al.*). However, from the 20th day, we noted a connective tissue which partially separates the gonad and the mesonephric tissue to prevent the cell migration and other substances, with this separation becoming more evident on the 23rd day, as described by Hayashi *et al*. (2000).

The onset of sexual differentiation in the New Zealand rabbits was observed from the 16th day of embryonic development (Mario *et al*., 2018), while in chinchillas it was reported around the 15th day (Díaz-Hernández *et al*.), in the mouse on the 13th day (Theiler, 1989); and in humans at 6 and 9 weeks (Bendsen *et al*., 2003). This differentiation was proven by the presence of the Dhh protein in the gonadal parenchyma, coelomic epithelium and sustentacular cells in embryos at16 days of gestational age to adulthood.

The Hedgehog protein family is the first gene associated with the onset of the differentiation of male gonads and regulates testicular morphogenesis and interstitial cells, in addition to having functions in myoid cells and allowing survival of spermatozoa in the adult testicle (Wilhelm *et al*.), as reported before for mouse (Bitgood *et al*., 1996), humans (Canto *et al*., 2005) and rats (Mäkelä *et al*., 2011). In males, suppression of Dhh culminates in spermatogenic modifications and infertility (Mäkelä *et al*.), while its absence in females does not alter gonadal development (Mario *et al*.) and fertility, and thus does not represent an important gene for ovarian development, although it has been demonstrated in granulosa cells from preantral and antral follicles (Spicer *et al*., 2009).

The morphogenesis of the seminiferous tubules in rabbits, as in humans, last several days. Therefore, only from the 18th gestational days was it observed using histological sections with clear organization of testicular cords, composed of mesonephric epithelial and mesenchymal cells surrounded by a basal lamina being followed by the organized seminiferous tubules. By contrast, in the mouse, seminiferous tubules form more rapidly and based on the aggregation of cells of the gonadal stroma, e.g., blood vessels, fibroblasts and precursors of myoid cells (Díaz-Hernández *et al*.).

The ducts system was observed in rabbits at around 14 and 20 days of embryonic development, similar to that reported in chinchillas (Díaz-Hernández *et al*.) and at 10 gestational weeks (Shen *et al*., 2018). The ducts were initially presented in mesonephroi arranged in a parallel way. The paramesonephric ducts are responsible for the development of the female ducts, while the mesonephric ducts develop the male reproductive tract (Wilhelm *et al*.), such as efferent ducts, ductus deferens and epididymis, which were visualized in this study from 20 and 23 days, respectively. The anti-Mullerian hormone (AMH), produced by sustentacular cells, allows for the regression of paramesonephric ducts and starts the sexual differentiation of male gonads, as stated in rabbit from the 15th day of gestation (Díaz-Hernández *et al*.).

The use of gonadal features as a criterion for sexual determination is controversial; however, we observed male fetus of rabbits from the 23rd day with larger gonads with an ovoid shape in contrast to the elliptical shape of female gonads (Mario *et al*.), as reported in rabbits by Nielsen & Torday (1983). Such features have also been described in the human fetus during 8 and 22 weeks of gestation (Shen *et al*.).

External genital differentiation in rabbits was observed from 23 days of fetal development from the anogenital distance, where the male fetus has a longer perineum than the female, which exhibited differentiated genitalia from the 24th day (Mario *et al*.). In humans, sexual differentiation occurs at around 17-18 weeks of gestation from the growing and differentiation of the external genitalia in the penis and clitoris (Shen *et al*.).

Anogenital distance is often used as a biomarker of natural variation in prenatal androgenization, and used for sexual determination in rabbits (Bánszegi *et al*., 2012) once the localization of penis is determined in animal at term, as its observation is difficult before such a period (Nielsen & Torday). However such distance can vary between genders in several species of rodents (Drickamer, 1996) and humans (Salazar-Martinez *et al*. 2004) or even in the same gender, depending on the amount of testosterone that the embryo has during the intrauterine period, which can be influenced by such variants as maternal stress and intrauterine position of fetus, as described in rodents (Drickamer; Bánszegi *et al*.).

Therefore, the study of the embryonic/fetus development of internal and external sexual structures in rabbits is important to understand congenital malformations and anomalies during biological mechanisms of sexual differentiation that commonly occur in humans and may present clinical application without serious consequences or even depending on surgical approaches to life maintenance (Shen *et al*.).

CONCLUSION

In summary, the features of sexual differentiation of embryos and fetuses of New Zealand rabbits were for the first time determined by morphological particularities and identification of the Dhh protein in embryos from 16 days of development to adulthood, which regulates testicular morphogenesis, in addition of description of the onset of the organization of seminiferous tubules and differentiation of external genitalia from 18 and 23 days, respectively.

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RESUMEN: El conejo se considera un modelo animal ideal para estudios que describen anomalías a nivel testícular debido a que presenta mecanismos morfogenéticos similares al desarrollo sexual y enfermedades que se encuentran comúnmente en los seres humanos. El objetivo de este estudio fue determinar la diferenciación sexual masculina del conejo de Nueva Zelanda (*Oryctolagus cuniculus*) a través del desarrollo. La edad gestacional se estimó y clasificó en 9, 12, 14, 16, 18, 20, 23 y 28 días gestacionales. La determinación morfológica y sexual se realizó mediante análisis histológico del tracto reproductivo en los embriones y fetos (9 - 28 días) así como mediante inmunohistoquímica -Desert hedgehog-Dhh- (proteína testicular específica en el cromosoma Y- 16, 20, 23 días y conejos adultos). Las gónadas se observaron a partir del día 14 en un estadio indiferenciado y con aspecto homogéneo. Se observó diferenciación sexual a partir del día 16 con presencia de células formadoras de cordones gonadales y células Dhh+ en el parénquima gonadal. A partir del día 18 de gestación se observaron cordones testiculares, que evolucionaron a túbulos seminíferos organizados. La formación de los conductos eferentes, deferentes y del epidídimo se observó a los 20 y 23 días, respectivamente. La diferenciación de los genitales externos ocurrió a partir del día 23 desde la distancia anogenital y se utilizó para identificar las estructuras del pene. En conclusión, las características de la diferenciación sexual se determinaron mediante la observación de la proteína Dhh en embriones desde el día 16 hasta la edad adulta, y las particularidades morfológicas observadas a partir del día 18 de gestación, determinadas por diferenciación de los genitales externos a partir del día 23.

PALABRAS CLAVE: Gónadas; Desarrollo embrionario; Túbulos seminíferos; Desert hedgehog; Células sustentaculares; Células germinales primordiales.

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