UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA

Ana Paula Ferragut Cardoso

Cryptorchidism and Orchiopexy in the rat: a possible model to study testicular dysgenesis syndrome (TDS)

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutora em Patologia

Orientador: Prof. Dr. Samuel Monroe Cohen
Coorientadorer: Prof. Dr. João Lauro Viana de Camargo
Profa. Dra. Merielen Garcia Nascimeno e Pontes

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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Cardoso, Ana Paula Ferragut.
Cryptorchidism and orchiopexy in the rat : a possible model to study testicular dysgenesis syndrome (TDS) / Ana Paula Ferragut Cardoso. - Botucatu, 2015

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina de Botucatu
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Coorientador: Merielen Garcia Nascimento e Pontes
Capes: 40105008

1. Criptorquidismo. 2. Orquidopexia. 3. Testículos - Métodos experimentais.

Palavras-chave: Cryptorchidism; Experimental model; Orchiopexy; Testicular dysgenesis syndrome.

Dedicatória

À minha família, meu suporte e a grande responsável pela minha trajetória até aqui, eu dedico este trabalho.

Agradecimentos

À Deus, eu dirijo minha maior gratidão.

Aos meus pais Roberto e Maria, que sempre acreditaram em mim e me apontaram o caminho certo. Obrigada por serem minha referência.

Ao meu irmão Danilo, pelo incentivo e amizade. Por estar comigo sempre e por saber que sempre estará.

Á Buzuza e Perdiz, que estão comigo desde que cheguei a Botucatu, sempre me apoiando, motivando e me ensinando a ser uma pessoa melhor.

Á Mariana e Nathália, pela amizade sincera de anos.

Á Viviane, por dividirmos não só uma mesinha no laboratório, mas também nossos melhores e piores momentos. É muito bom saber que você sempre vai estar comigo.

À Lígia, Nathália e Rafa, pela companhia ao longo dos anos, e pela amizade que certamente se perpetuará.

To my friend Karen Pennington – friends forever, facing whatever.

Aos amigos que ganhei durante o ano em Omaha e que me ajudaram muito enquanto estive lá. Vocês todos foram indispensáveis para que eu concluísse meu objetivo, me dando força e fazendo meus dias mais leves e felizes.

Á Cristina, pela ajuda essencial em todos os momentos.

To Puttappa, Sajana and Aparajita, my Indian friends.

Aos técnicos Paulo, Mara e PC que possibilitaram a realização deste trabalho.

À Coordenação de aperfeiçoamento de pessoal de nível superior, pelo suporte financeiro e pela concessão da bolsa do programa PDSE (Processo 001280/2014-07).

Á FAPESP, pelo auxílio financeiro (Processo 2012/09873-4).

Ao Núcleo de Avaliação do Impacto Ambiental Sobre a Saúde Humana, TOXICAM, pelo suporte na realização deste trabalho.

Á todas as pessoas que contribuíram para que este trabalho fosse concluído, meu muito obrigada!

Agradecimentos Especias

Ao Dr. João Lauro Viana de Camargo, pelos ensinamentos e pelo exemplo de dedicação à ciência.

To Dr. Samuel Cohen, who was more than generous sharing his knowledge and expertise.

À Dra. Merielen Garcia Nascimento e Pontes, pela co-orientação na realização deste trabalho. Obrigada pela paciência e amizade!

To Lora Arnold, for her encouragement, patient and friendship. Thanks for everything you have done for me.

À Dr Maria Luiza Cotrim Sartor de Oliveira pelos ensinamentos, apoio e amizade.

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Resumo

O criptorquidismo é uma anomalia congênito que afeta de 2 a 4% de meninos recém-nascidos, sendo um importante fator de risco para a infertilidade e tumores testiculares de células germinativas (TTCG). A cirurgia que realoca o testículo no escroto, oquidopexia, deve ser realizada entre 6 e 12 meses de idade, a fim de permitir fertilidade normal e reduzir o risco de malignidade. má qualidade do sêmen, testículo não descido, hipospadia e TTCG são elementos que, isolados ou combinados, compõem a chamada síndrome de disgenesia testicular (SDT). O presente estudo teve como objetivo caracterizar as alterações testiculares induzidas por uma modelo experimental de criptorquidia e orquidopexia em ratos, com o eventual objetivo de melhor compreender a SDT. Método mecânico aperfeiçoado de indução criptorquidismo abdominal e uma variação na instalação da orquidopexia ancorando os testículos, e não a cauda do epidídimo, na superfície interna da parede do escroto e puxando-os para o escroto, são apresentados. Para isso, ratos machos Sprague-Dawley foram submetidos cirurgicamente a criptorquidismo, ancorando a tunica albugínea dos testículos à parede abdominal - o passo crítico do presente método - na 3ª semana de idade; alguns deles foram sacrificados após 3, 6 ou 11 semanas desta cirurgia, a fim de registrar a progressão morfológica das alterações testiculares induzidas pela criptorquidia. Outros animais criptorquídicos foram submetidos à orquidopexia após 3, 5 ou 9 semanas da criptorquidia; estes animais foram sacrificados 3 ou 8 semanas após orquidopexia. Animais falsamente operados (sham) foram submetidos a criptorquidia e orquidopexia nos mesmos momentos que os grupos cirúrgicos. Pelo menos dez e cinco animais foram utilizados em grupos de cirurgia e sham, respectivamente. Testículos criptorquídicos mostraram diminuição do peso, perda de células germinativas, parada na espermatogênese, apoptose e, eventualmente, alguns túbulos com padrão de somente

células de Sertoli, resultados já descritos com outros métodos mecânicos utilizados para induzir a criptorquidia. Três semanas após orquidopexia, o peso testicular ainda permaneceram diminuídos, mas a espermatogênese foi parcialmente recuperada. Após oito semanas da orquidopexia, foi observada uma restauração completa da espermatogênese, no entanto, os pesos dos testículos ainda esteve reduzido. Os métodos propostos para induzir a criptorquidia e a orquidopexia em ratos e o intervalo para a recuperação testicular são úteis para estudar alterações testiculares, particularmente às relacionadas com a SDT. No presente estudo, oito semanas de recuperação se mostrou um ótimo intervalo para restaurar o epitélio germinativo; no entanto, o momento ideal para realizar a orquidopexia não foi determinado uma vez que as respostas de recuperação testicular foi semelhante quando a orquidopexia foi induzida após 3, 5 ou 9 semanas da criptorquidia.

1. REVIEW OF THE LITERATURE

1.1. Abstract

Cryptorchidism is a congenital defect affecting 2-4% of newborn boys, and is a major risk factor for infertility and testicular germ cell tumors (TGCT). The surgery that reallocates the testis into the scrotum, ochiopexy, should be performed between 6–12 months of age in order to allow normal fertility and reduce the risk of malignancy. Poor semen quality, undescended testis, hypospadias and TGCT are elements that, isolated or combined, compose the so-called Testicular Dysgenesis Syndrome (TDS). The present study aimed to characterize the testicular changes induced by a cryptorchidism and orchiopexy experimental model in the rat with the eventual purpose of better understanding TDS. Improved mechanical methods of abdominal cryptorchidism induction and a variation in the orchiopexy installation by anchoring the testes, and not the cauda epididymis, into the internal surface of the scrotal wall and gently pulling

them down to the scrotum, are presented. To accomplish that, Sprague-Dawley male rats were surgically submitted to cryptorchidism by anchoring the testicular albuginea to the abdominal wall - the critical step of the present method - at 3 weeks of age; some of them were euthanized 3, 6 or 11 weeks after this surgery in order to register the morphological progression of cryptorchidism-induced testicular alterations. Other cryptorchidic animals were submitted to orchiopexy 3, 5 or 9 weeks after cryptorchidism; these animals were euthanized 3 or 8 weeks after orchiopexy. Sham operated animals underwent to cryptorchidism and orchiopexy at the same times as the surgical groups. At least 10 and 5 animals were used in surgical and sham groups, respectively, at the respective euthanasia moments. Cryptorchidic testis showed decreased weights, germ cell loss, spermatogenesis disruption, apoptosis and eventually some tubules with a Sertoli cells-only pattern, findings already described with other

mechanical methods used to induce cryptorchidism. Three weeks after orchiopexy, testes weights were still decreased, but spermatogenesis was partially recovered. After eight weeks, a complete restoration of spermatogenesis was observed; however, testes weights were still reduced. The proposed methods to induce cryptorchidism and orchiopexy in rats and a defined interval length for recovery are useful to study testicular alterations, particularly those related to TDS. In the present study, eight weeks of recovery was optimal to restore the germinative epithelium; however, an ideal time to perform the orchiopexy was not determined since testicular recuperation responses were similar when the 3rd, 5th, and 9th weeks after orchiopexy induction were compared.

1.2. Testicular dysgenesis syndrome

A dysgenetic testis is described as a testis with abnormal cell organization originating from atypical embryonic development. Cryptorchidism, hypospadia, testicular cancer, and infertility are associated with testicular dysgenesis and share many predisposing factors, such as maternal life style, and are themselves risk factors for each other (Sharpe & Skakkebaek, 2008; Toppari et al., 2010). These conditions can occur isolated or in combination with a common origin in fetal life, comprising the testicular dysgenesis syndrome (TDS) (Skakkebaek et al., 2001; Sharpe & Skakkebaek, 2008). It has been proposed that TDS may be caused by genetic and/or environmental factors, resulting in abnormal function of Sertoli and Leydig cells in fetal life with either immediate (altered testis descent, masculinization) or delayed (low sperm counts, testis cancer) consequences (Figure 1) (Sharpe et al., 2003).

Men with a history of cryptorchidism are frequently subfertile in adulthood, that condition being responsible for 20% of all azospermic patients. Despite orchiopexy, it has been reported that infertility occurs in one third of the cryptorchidic patients (Cortes

et al., 2003; Foresta et al., 2008).

Hypospadia is a condition where the urethral meatus opens in an abnormal location along the penis (Toppari et al., 2001). Increased incidences of hypospadia have been reported in Australia, Europe and in the United States (Nassar et al., 2007).

Testicular germ cell tumor (TGCT) is the most common malignancy in young men aged 15–34 years (Ferguson & Agoulnik, 2013). The incidence of TGCT varies widely between populations and is much higher in individuals of European descent than in those of African ancestries (Chung et al., 2013). TGCT can be classified into two main histological subgroups: classical seminoma, which resembles the primary germ cells from which they are derived, and non-seminomas, which display varying types of tissue differentiation, from embryonal carcinoma through teratoma, including tumors with mixed histology (Horwich et al., 2006; Turnbull & Rahman, 2011). TGCT are believed to arise via a pre-invasive phase of carcinoma *in situ* (CIS), originating from embryonic/fetal primordial germ cells (PGCs)/gonocytes arrested in their differentiation (Skakkebaek, 1972). These cells with blocked or delayed differentiation are vulnerable and may undergo abnormal cell divisions, increasing genetic instability. Thus, gene expression patterns in TGCTs show the closest similarity to PGCs and gonocytes (Gilbert et al., 2011; Boublikova et al., 2014).

Cryptorchidic boys are at high risk of testicular cancer with a relative risk of 3.7-7.5 times higher than the general population. Conversely, 5-10% of men who develop testicular cancer were or are cryptorchid (Thorup et al., 2010; McGlynn & Trabert et al., 2012). In addition to this risk factor, recently reported genome-wide association studies (GWAS) using samples from 1643 cases of TGCT and 8403 controls from the United Kingdom, implicate certain genes that predispose to TGCT development (Rapley et al., 2009; Turnbull et al., 2009; Gilbert et al., 2011).

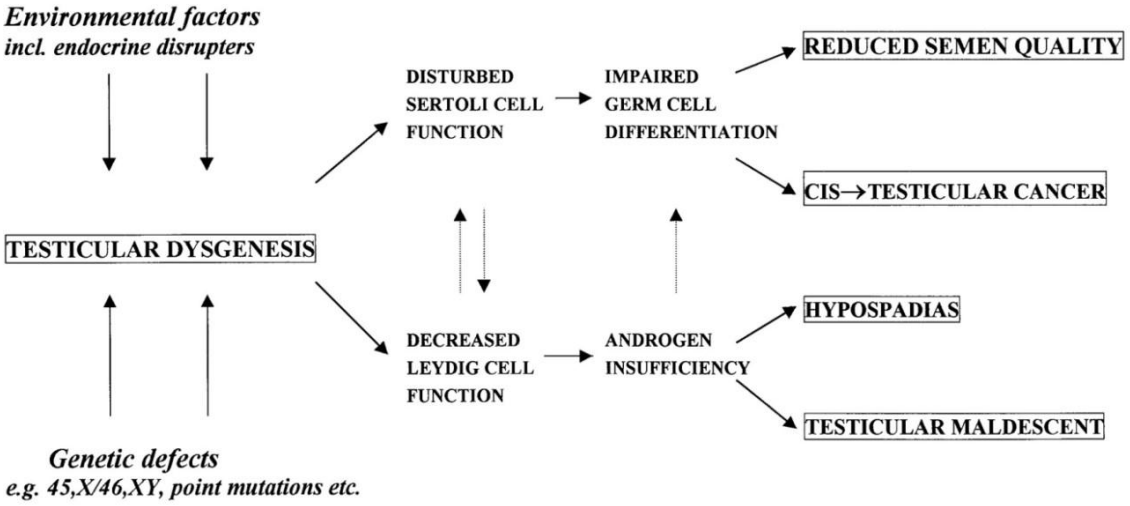

Figure 1: Schematic representation of the possible etiology, pathogenesis and clinical manifestations of testicular dysgenesis syndrome (Skakkebaek et al., 2001).

Development of TGCT may involve aberrantly activated KITLG/KIT pathway and overexpression of embryonic transcription factors such as NANOG and OCT3/4, which lead to suppression of apoptosis, increased cell proliferation, and accumulation of mutations in gonocytes. This distinct gene expression profile is likely caused by epigenetic regulation, in particular, DNA methylation (Sheikene et al., 2012).

1.3. Testis development

In humans, development of the undifferentiated gonads starts in both genders during the 5th and 6th weeks after fertilization, when germ cells migrating from the yolk sac arrive in the genital ridge, the mesenchyme just medial to the mesonephros. Between the 6th and 9th weeks, the metanephros, the final kidney, enlarges and ascends from the sacral region to a lumbar site just below the suprarenal gland. This movement results in lateral displacement of the gonad, caudaly to the final kidney. In mammals, the

initiation of the male pathway depends on gonadal expression of *Sry, a* Y-linked gene (Larsen, 2001; Virtanen et al., 2007).

Sry expression induces mesenchymal cells in the 7- to 8-week old ambisexual gonads to form Sertoli cells, the onset of sexual differentiation creating the testicular cords. Sertoli cells aggregate around germ cells and reorganize the gonad into two compartments: the tubular testis cords, composed of Sertoli and germ cells, and the interstitial space between the cords. The anti-Müllerian hormone (AMH) secreted by Sertoli cells around the 8th week causes regression of the Müllerian ducts. By the end of week 9, interstitial cell types arise and differentiate into Leydig cells which produce testosterone and induce both the differentiation of the Wolffian ducts into male internal accessory reproductive organs, and masculinization of the external genitalia after being converted to dihydrotestosterone (DHT) (Hughes, 2001; Hutson, 2005).

From about the 13th week the testis is anchored to the internal inguinal ring by the gubernaculum. The differentiation of fetal gonocytes into spermatogonia begins around gestational weeks 13–15 with downregulation of some stem cell markers (e.g., OCT-3/4 and KIT) and expression of additional germ cell specific proteins (Barteczko & Jacob, 2000; Rajpert-De Meyts, 2006).

1.4. Testicular descent

The testicular descent process can be categorized into two stages: the transabdominal and the inguinoscrotal. The transabdominal stage is characterized by the descent of the testis into the lower abdomen; during the inguinoscrotal stage the testis moves through the inguinal canal into the scrotum (Hutson et al., 1997). This phase is marked by gubernaculum enlargement and cranial suspensory ligament regression that keeps the testis anchored into the inguinal area; the second phase requires migration of

the gubernaculum and testis from the inguinal area into the scrotum (Hutson et al., 2010).

In humans the first stage of testicular descent occurs between the 10th and 15th weeks of gestation. Studies in mice have identified insulin-like hormone 3 (INSL3) as the major factor in transabdominal testicular descent (Ferguson & Agoulnik, 2013). *In vitro* studies of the fetal rat gubernaculum showed that INSL3 stimulates gubernacular growth, with both anti-Müllerian hormone (AMH) and testosterone providing some augmented stimulus (Hutson et al., 2015). The inguinoscrotal phase is clearly androgen-dependent and occurs between the 25th and 35th weeks of gestation in humans. Most effects of androgens are indirect, via the genitofemoral nerve in rodents, with the sensory fibers releasing calcitonin gene-related peptide (CGRP). This neurotransmitter appears to provide a chemotactic gradient to guide migration. The exact mechanism for the inguinoscrotal testicular descent is not clear in humans, but some studies are consistent with the CGRP possibility (Hutson et al., 2000; Hutson & Hasthorpe, 2005).

In rodents, the transabdominal phase occurs between gestational days 13-17 while the inguinoscrotal phase occurs up to the 21-28th days after birth. Apart from these differences in timing, the anatomy and hormonal regulation of the two stages of testicular descent are remarkably similar between rodents and humans (Hutson et al., 2015).

1.5. Testis histology

The testis is covered by a fibrous capsule, the tunica albuginea, composed of an inner basement membrane surrounded by a layer of myoid peritubular cells and displays two major compartments: the interstitium and the seminiferous tubules. The interstitial compartment contains the blood and lymphatic vessels and the quite frequent cell type,

Leydig cells, which are the major source of androgen, notably testosterone and other steroids (Russel et al., 1990). Seminiferous tubules are convoluted loops lined by the germinal epithelium and connected to the rete testis. They are bounded by the tunica propria where contractile myoid cells provide the major force for the movement of fluid and propulsion of sperm through the seminiferous tubules. The seminiferous epithelium consists of somatic supporting cells, the Sertoli cells, and a differentiating population of germ cells in various stages of maturation. As the germ cells mature, they move toward the lumen where they are released and transported into the rete testis (Haschek & Rousseaux, 1998). Sertoli cells are elaborately equipped to support spermatogenesis and to maintain the integrity of the seminiferous epithelium. Their functions include regulation of spermatogenesis, structural and metabolic support of the germ cells, spermiation, secretion of tubular fluid for sperm transport, and maintenance of a permeability barrier between the interstitial and tubular compartments. Sertoli cells and the germ cells are in contact with the basement membrane (Haschek & Rousseaux, 1998).

Spermatogenesis is the process by which primitive spermatogonia develop into highly specialized spermatozoa. This process may be divided into three phases: the proliferative, in which spermatogonia undergo rapid successive divisions, followed by the meiotic, in which spermatocyte genetic material is recombined and segregated, and subsequent differentiation, when spermatids transform into cells structurally equipped to reach and fertilize the egg (Russel et al., 1990). The process is fueled by stem cells that, when dividing, either self-renew or produce spermatogonia. The pituitary gland, under the influence of hypothalamic stimulation, secretes two mayor glycoprotein hormones that promote spermatogenesis, luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH has an indirect effect on spermatogenesis via Leydig

cell stimulation. These cells contain surface receptors for LH (LHR) and respond to stimulation by producing and releasing testosterone. Testosterone acts as a paracrine hormone in the testis, playing a critical role in the support of spermatogenesis; receptors for testosterone are present in the Sertoli cells which synthesize the transport protein, androgen-binding protein, for sequestering and transporting testosterone and its metabolite, dihydrotestosterone (Haschek & Rousseaux, 1998). Adult Leydig cells also synthesize and secrete estradiol, which can downregulate Leydig cell testosterone production, acting as an autocrine hormone. FSH directly stimulates the seminiferous tubule; its importance in initiation of spermatogenesis during pubertal development is well recognized. FSH acts directly on the Sertoli cells to stimulate germ cell number and acts indirectly to increase androgen production by the Leydig cells. Sertoli cells produce inhibin, which selectively inhibits the secretion of FSH (O'Shaughnessy et al., 2010).

1.6. Cryptorchidism and orchiopexy

Cryptorchidism, or undescended testis, is a congenital anomaly of newborn males, in which one or both testes are absent from the scrotum at birth. Around 2-4% of boys globally are diagnosed with cryptorchidism, but in approximately 70% of the affected infants the testes spontaneously descend during the first 3 to 6 months after birth (Rubenwolf & Stein, 2013). The high temperature in the abdominal cavity seems to be responsible for the degeneration of the seminiferous epithelium frequently seen in undescended testis (Mieusset et al., 1993).

The cause of undescended testis is believed to be multifactorial; however, disturbances of prenatal androgen secretion secondary to either deficient pituitary gonadotropin stimulation or low production of gonadotropin by the placenta are the

most relevant causes linked with cryptorchidism (Hutson et al., 1997). Some studies suggest genetic, environmental and social factors, and geographical differences affect the incidence of cryptorchidism. In Denmark, 9% of newborn males were diagnosed with this anomaly compared to only 2.4% in Finland. However, the recent quite significant increase in cryptorchidism incidences cannot be explained by genetic factors alone. Environmental and lifestyle factors must also be considered (Boisen et al., 2004; Thorup et al., 2010).

The principal consequences of cryptorchidism are infertility and testicular cancer. This malignancy may also originate in the contralateral, not retained, testis which can show some functional alterations, also. The main human testicular alterations variably seen in cryptorchidism are reduced number of germ cells, defective or delayed spermatogenesis, reduced number of Leydig cells, distorted seminiferous tubules, immature Sertoli cells and microcalcifications, indicating testicular dysgenesis (Skakkebaek et al., 2001; Cortes et al., 2006). Histological changes in the cryptorchid testes are variable depending on the age of the individual at the time of biopsy/orchidopexy and the position and duration of the testicular ectopia (Virtanen et al., 2007).

The optimal time to relocate the testes down to the scrotum (orchiopexy) remains controversial. Since the 1950's, the recommended period for this surgery has decreased from early adolescence to 6–12 months of age, as recently suggested (Hutson et al., 2013). The earliest relocation of the testis into the scrotal environment with its lower temperature will allow the germ cells to develop normally, and thus decrease the risk of oligospermia and cancer (Hutson et al., 2010).

1.7. Experimental cryptorchidism and orchiopexy

Experimental models of cryptorchidism in rodents have been used to investigate the etiology, pathophysiology and treatments for the disorder as well as to study infertility (Bergh & Soder, 2007). Undescended testis may be surgically induced (Nishimune et al., 1978; Kerr et al., 1979; Jégou et al., 1983; Quinlan et al., 1988; Shono et al., 1996; Dundar et al., 2001; Rossi et al., 2005; Garcia et al., 2011). Subcutaneously injected flutamide at 10 mg/100g of body weight into pregnant dams on the 16^{th} and the 17^{th} days of gestation can also interrupt the testicular descent (Spencer et al., 1991; Husmann & McPhaul, 1992). *In utero* exposure (embryonic days 13.5 to 21.5) to 500 mg/kg of dibutyl phthalate (DBP) by oral gavage has been shown to induce cryptorchidism, hypospadia, impaired spermatogenesis, and reduced fertility in male rats (Mylchreest et al., 2002; Mahood et al., 2005). Besides surgical and chemical interventions, natural and genetically engineered models are used to study undescended testis (Dundar et al., 2001).

The Long-Evans/Cryptorchid (LE/Orl) rat, a derivative of the Long-Evans strain, has been described as an animal model of naturally occurring cryptorchidism that maintains predictable patterns of maldescent and does not require surgical or hormonal manipulation to achieve cryptorchidism (Lugg et al., 1996; Mouhadjer et al., 1989). Transgenic mice overexpressing CYP450 aromatase and insulin-like 3, and knockout mice for the luteinizing hormone receptor (LHR) genes and the thyroid-specific enhancer/binding protein T/ebp/Nkx2.1, are models where cryptorchidism occurs as a single manifestation, or in combination with other abnormalities like disturbances in hormone balance (Huhtaniemi & Poutanen, 2004).

Different from the large number of cryptorchidism models, there are few orchiopexy rodent models, all involving surgical procedures, and used to evaluate

testicular recovery (Nelson, 1951; Jégou et al., 1983b; Seethalakshmi & Steinberger, 1983; Mizuno et al., 2008).

In rodents, cryptorchidism is associated with decreased testicular weight and spermatogenesis impairment due to germ cell loss (Morgentaler et al., 1999). Bilateral surgical cryptorchidism established in adult male Wistar rats of 4–5 months of age, and euthanized 3, 7, 20 or 28 days after the surgery, caused reduction of testes weights from the 3rd day after cryptorchidism. Associated with this alteration, germ cell removal from the seminiferous epithelium occurs in the following order: elongating spermatids and spermatids, round spermatids and elongated spermatids, and later involving also spermatocytes (Liu et al., 2012).

Sertoli and Leydig cells functions were also disrupted in 35-day old Sprague-Dawley rats that were made cryptorchidic at the 14th day of age (Jegou et al., 1983). Accumulation of lipid in Sertoli cells and local dilation of intercellular spaces between Sertoli cell junctions have also been reported. Leydig cell hypertrophy is accompanied by hyperplasia of smooth endoplasmic reticulum and of mitochondria, which are involved in steroidogenesis. In addition, blood flow and vascular permeability change (Bergh, 1989).

A number of studies have examined the ability of orchiopexy, performed at different time points, to restore spermatogenesis in the experimentally cryptorchid testes (Zini et al., 1998). The post-natal sexual development in the male rat has been classified into four phases: neonatal (post-natal day PND 1– 7), infantile (PND 8–21), juvenile (PND 22–35) and peripubertal (PND 36–55 or 60) (Clegg, 1960; Ojeda et al., 1990). Male rat puberty occurs around the 50th day of age, when the first spermatozoas are observed in the cauda epididymis and reproductive capacity is attained. Maximum sperm production is achieved at 75 days of age when male rats can be considered adult.

However, only at 100 days of age do the animals reach full sexual maturity, with maximum concentration of sperm stored in the cauda epididymis (Favaretto et al., 2011). Experimental data revealed that orchiopexy before sexual maturation of the testis restored spermatogenesis and reversal of germ cell degeneration, as demonstrated in 130-day old Wistar rats after cryptorchidism and orchiopexy induction at the 14th and 35th days, respectively (Karpe et al., 1981; Jegou et al., 1983b). In contrast, testis relocation in adult rats failed to restore normal spermatogenesis (Jegou et al., 1983). The timing of orchiopexy and its effect on the regeneration of the undescended testis is still controversial since a study did not observe any difference between performing orchiopexy before or after puberty (Patkowski et al., 1992). Therefore, it is important to clarify the influence of orchiopexy timing in an experimental cryptorchid rat model.

1.8. Apoptosis and cell proliferation in the testis

Apoptosis is the predominant mechanism of germ cell disappearance in cryptorchidism, rather than atrophy or necrosis (Koçak et al., 2002). Sprague Dawley rats showed a high number of germ cells in apoptosis after the second week post cryptorchidism induction, at 20-22 days of age. Besides that, a rise in apoptotic cell number was observed at week 16, after which the levels decreased until the 24th week (Watts et al., 2000). Caspases play a critical role in the execution of apoptosis in a number of cell types and can be classified as initiators (caspase-8, -9, and -10) or effectors (caspase-3, -6, and -7) (Villa et al., 1997; Nunez et al., 1998). Among the caspases, caspase-3 is a key protease in the apoptotic pathway. Activated caspase-3 targets DNA fragmentation factor (DFF), which is integrally involved in degrading DNA (Kim et al., 2000). Because caspase-3 is the main initiator of apoptosis, immunohistochemistry to detect the active form of caspase-3 has been run to check

apoptosis in paraffin sections from various tissues (Bressenot et al., 2009). Because of the importance of apoptosis, it has been postulated that the number of cells in a tissue does not depend on proliferation alone but rather on the quantitative relationship between the rates of cell proliferation and cell death. The seminiferous epithelium is a rapidly proliferating tissue and normally, a balance exists between these two processes, so that the number of cells neither increases nor decreases with time (Berges & Isaacs, 1993). Cryptorchidism is associated with a time-dependent decrease in germ cells, which indicates an imbalance between cell death and cell proliferation, resulting in a cell cycle severely impaired (Heiskanen et al., 1996).

1.9. Justification and Objective

Experimental models of cryptorchidism in rodents have been used to study the disorder and the resulting infertility (Bergh & Soder, 2007). Testicular dysgenesis syndrome (TDS) can be manifested as one or any combination of the following developmental abnormalities: cryptorchidism, hypospadia, reduced semen quality and testicular cancer (Ferguson & Agoulnik, 2013). There is not a comprehensive study that systematically characterizes the testicular changes caused by the available rat protocols of experimental cryptorchidism and orchiopexy. The present study aims to characterize the testicular changes, focused on the germinative epithelium, using a rat cryptorchidism and orchiopexy protocol with the eventual purpose of better understanding TDS pathogenesis.

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Manuscript

Time-response of rat testicular alterations induced by cryptorchidism and its reversibility

Ana Paula F. Cardoso[a], Ligia M. M. Gomide[a], Nathalia P. Souza[a], Carlos Márcio N. de Jesus[b], Lora L Arnold[c], Samuel M. Cohen[c], João Lauro V. de Camargo[a], Merielen G. Nascimento e Pontes[a].

[a]UNESP - São Paulo State University, Botucatu Medical School, Department of Pathology, Center for the Evaluation of the Environmental Impact on Humans Health (TOXICAM), Botucatu, São Paulo, Brazil.

[b]UNESP - São Paulo State University, Botucatu Medical School, Department of Urology, Botucatu, São Paulo, Brazil.

[c]UNMC - University of Nebraska Medical Center, Department of Pathology and Microbiology, Omaha, NE, USA.

Address correspondence to:

Dr. Merielen Garcia Nascimento e Pontes
Department of Pathology, Botucatu Medical School, UNESP – Univ Estadual Paulista,
Botucatu, 18618-970, São Paulo, Brazil
E-mail: merielen@fmb.unesp.br
Phone number: +55 14 38116238

Manuscript according to Author Guideline for submission on *Acta Paediatrica* ISSN 0803-5253 (Print), ISSN 1651-2227 (Online). Impact factor (2013): 1,842.

3. ABSTRACT

Cryptorchidism is a congenital defect affecting 2-4% of newborn boys, and is a major risk factor for infertility and testicular germ cell tumors (TGCT). The surgery that reallocates the testis into the scrotum, ochiopexy, should be performed between 6–12 months of age in order to allow normal fertility and reduce the risk of malignancy. Poor semen quality, undescended testis, hypospadias and TGCT are elements that, isolated or combined, compose the so-called Testicular Dysgenesis Syndrome (TDS). The present study aimed to characterize the testicular changes induced by a cryptorchidism and orchiopexy experimental model in the rat with the eventual purpose of better understanding TDS. Improved mechanical methods of abdominal cryptorchidism induction and a variation in the orchiopexy installation by anchoring the testes, and not the cauda epididymis, into the internal surface of the scrotal wall and gently pulling them down to the scrotum, are presented. To accomplish that, Sprague-Dawley male rats were surgically submitted to cryptorchidism by anchoring the testicular albuginea to the abdominal wall - the critical step of the present method - at 3 weeks of age; some of them were euthanized 3, 6 or 11 weeks after this surgery in order to register the morphological progression of cryptorchidism-induced testicular alterations. Other cryptorchidic animals were submitted to orchiopexy 3, 5 or 9 weeks after cryptorchidism; these animals were euthanized 3 or 8 weeks after orchiopexy. Sham operated animals underwent to cryptorchidism and orchiopexy at the same times as the surgical groups. At least 10 and 5 animals were used in surgical and sham groups, respectively, at the respective euthanasia moments. Cryptorchidic testis showed decreased weights, germ cell loss, spermatogenesis disruption, apoptosis and eventually some tubules with a Sertoli cells-only pattern, findings already described with other

mechanical methods used to induce cryptorchidism. Three weeks after orchiopexy, testes weights were still decreased, but spermatogenesis was partially recovered. After eight weeks, a complete restoration of spermatogenesis was observed; however, testes weights were still reduced. The proposed methods to induce cryptorchidism and orchiopexy in rats and a defined interval length for recovery are useful to study testicular alterations, particularly those related to TDS. In the present study, eight weeks of recovery was optimal to restore the germinative epithelium; however, an ideal time to perform the orchiopexy was not determined since testicular recuperation responses were similar when the 3rd, 5th, and 9th weeks after orchiopexy induction were compared.

4. INTRODUCTION

Failure of testicular descent, or cryptorchidism, is a common anomaly that affects 2%-4% of newborn boys. The ectopic testes are most frequently found in the inguinal canal or upper scrotum; arrest within the abdomen is less frequent. In approximately 18% of cases both testes are cryptorchid (Bostwick & Cheng, 2008; Ferguson & Agoulnik, 2013). Cryptorchidism is associated with impairment of germ cell maturation, subsequent infertility and testicular germ cell tumors (TGCT) (Bostwick & Cheng, 2008). It has been proposed that these disorders, including hypospadia, may comprise the testicular dysgenesis syndrome (TDS) with a hypothetic common origin during fetal life, possibly resulting from the influence of environmental and or parental life-style factors (Skakkebaek et al., 2001; Sharpe & Skakkebaek, 2008). Several epidemiological studies have shown that these conditions - cryptorchidism, impaired spermatogenesis, hypospadias and testicular cancer - can be associated as mutual risk factors (Virtanen et al., 2007). Thus, approximately 10% of all cases of testicular germ cell tumors occur in men with a history of cryptorchidism, making this disorder a primary risk factor for TGCT

development (Mannuel et al., 2012; Banks et al., 2013). In order to allow normal fertility and prevent malignancy, the surgery to reallocate the testes to the scrotum – orchiopexy – should be performed between 6 and 12 months of age (Hutson et al., 2013).

Different experimental models have been used to study cryptorchidism in rodents. These models have provided a better understanding of the physiopathology and potential treatments for cryptorchidism (Watts et al., 2000; Bergh & Soder, 2007). A National Toxicology Program (NTP) task force emphasized that the presence of cryptorchidism and low sperm counts in rodents might be useful as early predictors of testicular germ cell tumor induction (Thayer & Foster, 2007).

Undescended testis may be surgically induced (Jégou et al., 1983; Rossi et al., 2005; Garcia et al., 2011) by fixing the epididymidis' tail or the paratesticular adipose tissue to the abdominal wall. However, these methods can induce spermatic cord torsion and are not precise regarding the testicular position, which is crucial for accurate data interpretation. Exogenous hormones such as estradiol (Lein et al., 1996) or anti-androgens like flutamide (Goh et al., 1993; Van der Schoot, 1992) and dibutyl phthalate (DBP) (Mylchreest & Foster, 2000; Mylchreest et al., 2002; Mahood et al., 2005) can also induce cryptorchidism. Besides surgical and chemical approaches, congenital and genetically engineered models have been used to study undescended testes (Dundar et al., 2001; Huhtaniemi & Poutanen, 2004).

The Long-Evans/Cryptorchid (LE/Orl) rat, a derivative of the Long-Evans strain, has been described as an animal model of naturally occurring cryptorchidism that maintains predictable patterns of maldescent and does not require surgical or hormonal manipulation to create a cryptorchidic animal (Mouhadjer et al., 1989; Lugg et al., 1996). Transgenic mice overexpressing the CYP450 aromatase and the insulin-like 3 (INSL-3) hormone, and the knockout mice for the luteinizing hormone receptor (LHR) genes and the

thyroid-specific enhancer/binding protein T/ebp/Nkx2.1 are models where cryptorchidism occurs as the single manifestation or in combination with other abnormalities like disturbances in hormone balance (Huhtaniemi & Poutanen, 2004). Different from the large number of cryptorchidism models, there are few orchidopexy rodent models, all involving surgical procedures (Nelson, 1951; Jégou et al., 1984; Seethalakshmi & Steinberger, 1983; Mizuno et al., 2008).

This laboratory has been developing efforts to establish a model of testicular susceptibility to environmental harmful influences, which can be used to better understand TDS. In order to establish that experimental model, we assessed mechanical methods to induce cryptorchidism and orchiopexy. The surgical procedures available in the literature with gubernaculectomy (Rossi et al., 2005) or suturing the paratesticular adipose tissue on the abdominal wall (Nishimune et al., 1975; Garcia et al., 2011) were not effective to induce cryptorchidia in the rats in the present study. Even after performing those surgeries, the testis returned to the scrotum. Herein, a variation in one of these mechanical methods is presented, based on suturing the testes albuginea, and not the paratesticular adipose tissue, to the abdominal wall with two stitches at the cranial and caudal regions respectively. For orchiopexy installation, we fixed the testes, and not the epididymis' tail (Jégou et al., 1984), into the everted scrotal wall, gently pulling them down into the scrotum. In addition, in order to verify the timing of orchiopexy and its effect on the regeneration of the undescended testis (Quinn et al., 1991; Mizuno et al., 2008), we assessed three different moments of orchiopexy induction and two intervals of recovery. The effectiveness of the cryptorchid and orchiopexy rat models and the influence of orchiopexy timing were evaluated by histological examination of the testes. The role of apoptosis was evaluated in immunohistochemically stained testicular sections and of cell proliferation in H&E sections, in order to document the balance between these two processes.

5. MATERIAL AND METHODS

5.1. Experimental outline

This experiment was approved by the Committee for Ethics in Animal Experimentation of the UNESP Medical School, SP, Brazil, protocol no. 926/2012 (Appendix). Sprague Dawley rats were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB, UNICAMP, Campinas, São Paulo, Brazil) and placed in the animal facility under a 12-h light/dark cycle and controlled temperature (22 ± 2 ºC). Standard pellet food (Presence; Evialis, Paulínia, SP, Brazil) and tap water were provided *ad libitum*. After a two-week acclimation period, adult female rats were mated overnight with two females to each male.

Three randomly established groups of 21-days old animals were surgically submitted to abdominal cryptorchidism, as described below. Animals from one group were euthanized three, six or eleven weeks after cryptorchidism induction: Groups CPT+3, CTP+6 and CPT+11, respectively. Another two groups were also submitted to orchiopexy after three (ORC3), five (ORC5) or nine (ORC9) weeks of cryptorchidism induction. Animals from these groups were euthanized either after three (Groups ORC3+3 ORC5+3 and ORC9+3) or eight (Groups ORC3+8, ORC5+8 and ORC9+8) weeks after orchiopexy. Sham animals were operated at the same moments as the cryptorchidism or orchiopexy surgical procedures (Figure 1). For each surgical and sham groups, at least 10 and 5 animals were used, respectively.

For euthanasia, the rats were anesthetized with ketamine (30 mg/kg ip) and xylazine (4 mg/kg ip) between 8:00 and 10:00 a.m. and submitted to exsanguination via heart puncture. The testes and epididymes were removed, weighed, and placed in modified Davidson's fixative for 24h (Latendresse et al. 2002; Kittel et al. 2004). The seminal

vesicles and ventral prostate were collected, weighed and fixed in 10% formalin. After fixation, all of the organs were embedded in paraffin.

5.2. Surgical techinique for cryptorchidism

The rats (weights varying between 60 to 70g) were anesthetized with ketamine (30 mg/kg i.p.) and xylazine (4 mg/kg i.p.); abdominal anesthesia and analgesic effects were achieved with local injections of lidocaine (7 mg/kg, sc) and ketoprofen (10 mg/kg, sc), respectively. Next, the abdominal region was shaved and cleansed with povidon iodine. The abdominal cavity was opened through a small midline incision and the testes were translocated from the scrotum into the abdomen through the inguinal rings using tweezers. Care was taken to avoid spermatic cord torsion, which could lead to testicular atrophy. Both testes were attached to the inner dorsolateral abdominal wall with two stitches at the cranial and caudal regions, respectively, with great care due to the fragility of the gonad, using a 5-0 blunt needle with nonabsorbable suture material (Nylon, Bioline, Fios Cirúrgicos LTDA, BR) passing through the tunica albuginea (Figure 2). This step is critical to assure the success of this model; the two stitches anchoring the albuginea into the abdominal wall guarantee the permanency of the testes in exactly the position desired and avoid the spermatic cord torsion and consequent atrophy of the ectopic gonad by the absence of an adequate vascular supply. This limitation was found in the gubernaculectomy procedure proposed by Rossi et. al (2005) which induces cryptorchidism by anchoring the gubernaculum into the abdominal wall. Another mechanical procedure described in the literature, which fixes the paratesticular adipose tissue, was also performed (Garcia et al., 2011), but the 21 days old rats used in the present study are in a major body weight gain period and the gubernacular traction was stronger than the paratesticular adipose fixation, resulting in the testes returning to the scrotum.

At the end of the surgery, the abdominal wall was closed with 5-0 triangular tipped needles with nonabsorbable suture material (Nylon, Bioline, Fios Cirúrgicos LTDA, BR). The abdomen was cleansed with povidon iodine due to its antiseptic function. Animals submitted to sham surgeries underwent the same pre-surgical procedures; their abdomens were similarly opened and closed by sutures through the muscular and skin layers.

5.3. Surgical techinique for orchiopexy

The anesthetic, analgesic and asepsis procedures were the same as for the cryptorchidism surgery. A midline abdominal incision was made and the sutures which held the testes to the posterior abdominal wall were carefully cut to avoid rupture of the tunica albuginea. Tweezers were introduced into the scrotum through the inguinal ring, so the inner surface of the scrotum wall was clamped and reversed to facilitate the manipulation. The testes were attached to the scrotum inner surface of the scrotum wall by a suture (5-0 blunt needle, nonabsorbable suture material, Nylon, Bioline, Fios Cirúrgicos Ltda., BR) passing through the tunica albuginea. Finally, using tweezers, the testes were guided into the scrotum (Figure 2). The abdominal wall was closed using 5-0 triangular tipped needles with nonabsorbable suture material (Nylon, Bioline, Fios Cirúrgicos LTDA, BR). The abdomen was cleansed with povidon iodine. Animals submitted to sham surgeries went through the same pre-surgical procedures and their abdomens were similarly surgically opened and closed. All of the animals received antibiotic (enrofloxacin 5 mg/kg sc) during the three days following surgery because this surgery was longer and more invasive than the procedures for cryptorchidism.

5.4. Histological examination

Immediately after euthanasia the testes were removed, weighed, and placed in modified Davidson's fixative (Kittel et al., 2004). Afterwards, they were embedded in paraffin, cut in histological sections of 5 μm thickness and stained with hematoxylin and eosin (H&E). The histological analysis was systematically and blindly performed by one of the authors (APFC) using an optical microscope with 40x magnification. The whole section of testis was recorded. All round seminiferous tubules were classified in classes according to the most differentiated germ cell type predominating in the germinative epithelium: Class 1 - tubules showing normal spermatogenesis, containing spermatids and sperms; Class 2 - tubules with spermatids and sperms but also showing damage such as epithelial vacuoles, apoptotic bodies and/or giant germ cells; Class 3 - tubules presenting spermatocytes and spermatogonia with the same elements of epithelial damage; and Class 4 - Sertoli cells-only tubules (SCO). The Johnsen score (Johnsen, 1970) used to evaluate histologically the human testis was tentatively applied but it did not reflect the true status of the seminiferous tubules after the surgeries because it considers the cell type occurring within the germinative epithelium and not eventual alterations like vacuoles and other cell alterations. The four-classes classification system described above was developed by our laboratory based on the most frequent morphologic testicular alterations observed in H&E sections. The incidence of tubules (%) in each class was calculated by multiplying the number of tubules of that class times 100 and dividing by the total number of tubules counted in the section. In sham groups, the minimum, maximum and average of seminiferous tubules counted was 199, 592 and 434, respectively. In the surgery groups the minimum of tubules counted was 166, the maximum was 534 and the average was 358.

5.5 Apoptotic index

For caspase-3 immunohistochemical staining, testicular sections from five animals of the surgical groups and from three animals of sham groups were used. Tissues were deparaffinized, rehydrated, and blocked of endogenous peroxides. For antigen retrieval, tissue sections were placed in citrate buffer, pH 6.0 and microwaved for 8 min. Incubation in avidin/biotin blockers (Vector Laboratories, Burlingame, CA) and goat serum were performed to block nonspecific binding. Tissue sections were incubated overnight at approximately 4°C with the primary antibody (anti-caspase-3, rabbit monoclonal, Cell Signalling, Danvers, MA) diluted 1:10. The sections were then incubated with a biotinylated second antibody followed by incubation with a peroxidase-conjugated biotin-avidin complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). The presence of the peroxidase was detected by staining with DAB (DAB Substrate kit, Vector Laboratories, Burlingame, CA). Tissue sections were then counterstained with hematoxylin. The total number of positive cells and the total number of seminiferous tubules in the section were counted. The labeling index (LI) was calculated by dividing the number of caspase-positive cells per the number of seminiferous tubules x 100.

5.6. Mitotic index

Immunohistochemical analysis for Ki-67 could not be performed because of the use of modified Davidson's fixative. Therefore, the mitotic index (MI) was assessed on H&E sections. Five samples of each surgical group, and three of each sham group were analyzed. The MI was calculated by dividing the total number of cells undergoing mitosis by the total number of seminiferous tubules x 100.

5.7. Statistical analisis

A normality test (Kolmogorov-Smirnov) was performed, followed by Student's t test (parametric) or Mann-Whitney (non-parametric) for organ weights. For histological

analysis, the Fischer exact test was used, and for caspase-3 and the MI, the Rumk sum test was used. A p value < 0.05 was considered statistically significant. The groups were compared to their respective sham groups.

6. RESULTS

The cryptorchidism and orchiopexy surgery methods proposed were well tolerated by the animals - none of them died due to the surgical procedure and no evidence of infection was noted afterwards. In 10% of the cryptorchidic animals (4/39), one of the testes was atrophic and in 15% (6/39) one of the testes spontaneously returned to the scrotum. After orchiopexy, there was no adhesion between the abdominal wall and the testes; gonadal removal and reallocation into the scrotum was easily accomplished.

No significant differences were found in body weights between groups (*data not shown*). There was a significant decrease of absolute testes weights and epididymides relative weights in all surgical groups when compared to their respective sham animals (Table 1). Animals from cryptorchidic groups euthanized after three (CPT+3), six (CPT+6) or eleven (CPT+11) weeks of cryptorchidism surgery showed reductions ($p<0.05$) of 50%, 69% and 71% of the absolute testes weights, respectively, when compared to their respective sham groups. After three weeks of recovery (interval after orchiopexy), the testes weights of the ORC3+3, ORC5+3 and ORC9+3 were similar to those of the cryptorchidic groups (reduction about 60%) and still significantly ($p<0.05$) lower than their respective sham groups. However, after eight weeks of recovery (ORC3+8, ORC5+8 and ORC9+8), the absolute testes weights were decreased 20%, 25% and 41% when compared to the respective sham groups, respectively, pointing to partial testicular recovery. Despite

that weight replenishment, they were still significantly lower than their respective sham groups. The epididymides went through a similar process of weight loss and recovery (Table 1).

The CPT+11, ORC5+3, ORC9+3 and ORC9+8 groups showed a decrease ($p<0.05$) in the relative prostate weights. Only in the CPT+6 group was the seminal vesicle weight significantly decreased (Table 2).

All sham groups, irrespective of surgical operations and age at necropsies, presented normal tubules with complete spermatogenesis (Class 1, $p<0.001$). The histological analyses of all cryptorchidic groups showed a predominance of tubules containing spermatocytes and spermatogonia, vacuoles, apoptotic and giant cells (Class 3, $p<0.001$), and SCO tubules (Class 4, $p<0.001$). The incidences of SCO increased as the interval between cryptorchidism and euthanasia also increased. In the 3 week recovery groups after orchiopexy (ORC3+3, ORC5+3 and ORC9+3), the tubules showed a Class 3 pattern ($p<0.001$), and also spermatid and sperm predominance with vacuoles and apoptotic and giant cells (Class 2, $p<0.001$). After eight weeks of recovery, all three groups, ORC3+8, ORC5+8 and ORC9+8, presented tubules with normal spermatogenesis (Class 1, $p<0.001$) (Table 3, Figure 3).

Immunohistochemical detection of caspase-3 was significantly higher in all cryptorchidic groups, CPT+3, CPT+6 and CPT+11, when compared to their respective sham groups ($p<0.05$) (Figure 4), showing a LI around 18%, 15% and 5%, respectively, indicating apoptosis in progress in those groups. Cells expressing this apoptotic marker were still seen in the three week after orchiopexy recovery groups, LI varying from 4to 6%, not different from the respective sham groups. In the eighth week recovery after orchiopexy groups, the caspase-3 LI was low, below 4%, at similar levels as the respective sham groups (Figure 5).

The MIs were significantly decreased to less than 5% in all cryptorchidic groups, while in the sham groups it varied between 15-30%. Three weeks after orchiopexy, the MI was still below the respective control (5-15% and 20-30%, respectively), however, this difference was not statistically significant. After eight weeks of recovery after orchiopexy, the MIs in the operated groups were between 15-30%, similar to the levels observed in the sham group, 20-30% (Figure 4 and 6).

7. DISCUSSION

Experimental models have been used to study testicular alterations induced by cryptorchidism and characterize changes possibly associated with infertility (Watts et al., 2000). However, there is not a comprehensive study that systematically characterized the testicular changes caused by combined protocols of experimental cryptorchidism and orchiopexy. Our model of surgical cryptorchidism in the immature rat induced testicular changes similar to those seen in other experimental rat models of cryptorchidism: decrease in testicular weight, tubular atrophy, germ cell loss and spermatogenesis disruption (Jegou et al., 1983; Jegou et al., 1984; Zakaria et al., 1998; Rossi et al., 2005). After cryptorchidism, few testicular losses due to atrophy or descent were recorded, mainly in the beginning of the experiment, particularly due to the lack of training to perform such surgery. Once the experience is attained, this present model can be useful for the production of cryptorchidism and induction of testicular alterations. Moreover, our model for orchiopexy effectively induced germ cell replenishment and spermatogenesis recovery, as observed in other studies (Jégou et al., 1984).

The significant decrease of testis weight in all groups submitted to cryptorchidism may be related to the extensive germ cell loss and probably to a decreased production of testicular fluid by the Sertoli cells (Jegou et al., 1983). As the duration of cryptorchidism

was extended there was a progressive loss of germ cells and increased incidence of SCO tubules (Class 4). On the other hand, the number of germ cells expressing caspase-3 declined gradually as the interval between cryptorchidism and euthanasia increased. These findings suggest an early activation of caspase-3 and a gradual germ cell disappearance due to apoptosis, leading to an extensive germinative epithelium loss eleven weeks after cryptorchidism. Apoptosis is a common process during normal spermatogenesis in mammals, which can guarantee cellular homeostasis and a fine balance between the maturing germ cells and Sertoli cells (Kierszenbaum, 2001; Gautam et al., 2007). However, a variety of both physical and chemical stresses to the testis, such as trauma, heat, radiation, or withdrawal of hormonal support are shown to enhance the apoptotic process of germ cells (Lee et al., 1999, Gautam et al., 2007). Exposure of the rat testis to 6 hours of mild heat results in activation of apoptosis by a redistribution of Bax from a cytoplasmic to a paranuclear localization in heat-susceptible germ cells (Yamamoto et al., 2000). The relocation of Bax is associated with activation of the initiator caspase-9 and the executioner caspases-3, 6 and 7 (Hikim et al., 2003).

On the contrary, the mitotic indices (MI) were reduced in all three cryptorchidic groups, being more accentuated in the CPT+11 probably due to the increase in cell death and lack of germ cells. Spermatogonias are the major proliferative cells of the testes, showing high mitotic activity; decreased proliferation of spermatogonias could be due to alterations in the spermatogonias themselves or consequences of changes in the Sertoli cells (Bernal-Mañas et al., 2005). The frequent and conspicuous occurrence of vacuoles within the germinative epithelium suggests that the Sertoli cells were also severely compromised by the cryptorchidic condition (Kerr et al., 1979). The progressive return of the MIs to the levels of the sham-operated animals as the interval after the orchidopexy surgery increased, in parallel with decreased incidence of epithelial alterations, document

the potential recovery of the seminiferous tubules activity.

The most appropriate time to perform orchiopexy is still unclear, but it has been suggested that early orchiopexy surgery is preferable in preventing degeneration and enhancing recovery of the seminiferous epithelium (Quinn et al., 1998; Mizuno et al., 2008). However, most of the studies did not evaluate the interval for a complete reversibility of testicular damage caused by cryptorchidism. In our experimental study, orchiopexy was performed in the pre- and post-puberty ages. In addition, two intervals of recovery were evaluated. Animals left to recover for 3 weeks (ORC3+3, ORC5+3 and ORC9+3) showed partial recovery of the germinative epithelium with the presence of spermatids and sperm (Class 2), leading to an almost complete recovery of testes weights within this three-week interval. Despite some cells expressing caspase-3 and the slight recuperation of the MI in these groups, there were no differences compared to the sham group. The higher MIs representing proliferative cells, rather than apoptosis, explains the partial restoration of the seminiferous epithelium, with the presence of spermatids and sperm. The regeneration process naturally starts with proliferation of the remaining spermatogonial stem cells, leading to cellular replenishment. The interval of three weeks of recovery was not enough to induce a complete recuperation of the seminiferous tubules and spermatogeneis, or even a complete testis weight recovery, and there was no difference between the moments of performing the orchiopexy surgery.

However, eight weeks after orchiopexy, animals from all groups (ORC3+8, ORC5+8 and ORC9+8) showed increased testes weights, but still different from the sham groups. In these groups, the morphology of seminiferous tubules was similar to the sham group, with normal spermatogenesis, although some vacuoles and apoptotic bodies remained. Caspase-3 expression was very low and the MI returned to normal, both levels similar to sham groups, indicating an almost complete recovery of the seminiferous

tubules. Taking all of the parameters evaluated into consideration, in the eight week recovery groups there was no ideal time to perform the orchiopexy surgery since the testicular recuperation responses were similar between the 3rd, 5th, and 9th weeks of orchiopexy induction.

The decreased relative epididymal weight in the cryptorchidic groups and in those with 3 weeks of recovery could also be explained by the loss of sperm and decrease in the production of testicular fluid by Sertoli cells (Jegou et al., 1983; Jegou et al., 1984). Although animals from groups after eight weeks of recovery showed normal spermatogenesis, they still had a decrease in epididymal weight, probably due to not enough production of sperm since the testicular weight was not fully recovered as well.

In the present study, the proposed experimental protocol for cryptorchidism led to decreased testis and epididymis weights, in addition to germ cell loss, tubular atrophy and spermatogenesis disruption. Based on the evaluations performed, the germinative epithelium still showed morphological damage after three weeks of recovery, but not at eight weeks, making the interval between orchiopexy induction and euthanasia crucial to induce complete spermatogenesis restoration. However, the orchiopexy surgery at different ages – before or after puberty – did not influence the testicular recuperation response, indicating that age and sexual maturity were not relevant for producing germ cell restoration and normal spermatogenesis.

In humans, even after orchiopexy, infertility and an increased risk for development of testicular malignancy are still reported, conditions that comprise the TDS. It has been proposed that there is a fetal origin for this syndrome resulting from genetic and/or environmental factors, with immediate (cryptorchidism) or delayed (testicular cancer) consequences (Skakkebaek, 2001; Foresta el al., 2008,). In this way, testicular germ cell tumors can arise in the fetal period from primordial germ cells arrested in their

differentiation (Skakkebaek, 1972), and despite normal development of germ cells after orchiopexy, a few cells could already be arrested, undergoing abnormal cell divisions. This study documented the role of cryptorchidism in the TDS by the disruption of germ cell proliferation homeostasis, the most recognized key-event for the carcinogenic development. Further studies with sensitive markers for germ cell malignancy in experimental models like the present one are necessary to better understand the relation between cryptorchidism and testicular cancer. Thereby, the association between orchiopexy induction and interval of recovery in an experimental model of cryptorchidism can be useful to study testicular alterations, including those related to testicular dysgenesis syndrome.

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9. TABLES

Table 1: Absolute testes weights and relative epididymal weights in surgical and sham groups.

	Groups	Testis (g)			Epididymis (%)		
		n	Surgery	Sham	n	Surgery	Sham
Cryptorchidism	**CPT+3**	9/5	0.36 ± 0.07*	0.72 ± 0.05	14/5	0.04 ± 0.01*	0.05 ± 0.00
	CPT+6	11/5	0.44 ± 0.06*	1.41 ± 0.10	14/5	0.05 ± 0.01*	0.09 ± 0.01
	CPT+9	9/6	0.42 ± 0.01*	1.45 ± 0.03	10/5	0.04 ± 0.01*	0.11 ± 0.00
3 weeks of recovery	**ORC3+3**	7/4	0.54 ± 0.05*	1.33 ± 0.13	9/4	0.04 ± 0.00*	0.08 ± 0.00
	ORC5+3	12/4	0.54 ± 0.10*	1.40 ± 0.04	12/4	0.04 ± 0.01*	0.11 ± 0.01
	ORC9+3	10/5	0.57 ± 0.05*	1.49 ± 0.13	12/5	0.05 ± 0.01*	0.14 ± 0.02
8 weeks of recovery	**ORC3+8**	7/5	1.09 ± 0.15*	1.36 ± 0.05	7/5	0.07 ± 0.01*	0.10 ± 0.01
	ORC5+8	8/5	1.04 ± 0.20*	1.38 ± 0.07	9/5	0.07 ± 0.01*	0.11 ± 0.01
	ORC9+8	7/5	0.90 ± 0.13*	1.51 ± 0.21	11/5	0.05 ± 0.01*	0.11 ± 0.00

Values are expressed as mean ± standard deviation, t Test, *$p<0.05$, n: number of animals in surgery group/number of animals in sham group.

Table 2: Ventral prostate and seminal vesicle relative weights (%).

	Groups	Ventral prostate (%)			Seminal vesicle (%)		
		n	Surgery	Control	n	Surgery	Control
Cryptorchidism	**CPT+3**	14/5	0.04 ± 0.01	0.04 ± 0.01	14/5	0.02 ± 0.01	0.02 ± 0.01
	CPT+6	15/4	0.07 ± 0.00	0.08 ± 0.00	14/5	0.15 ± 0.04*	0.20 ± 0.04
	CPT+9	10/5	0.09 ± 0.01*	0.11 ± 0.00	10/5	0.22 ± 0.02	0.22 ± 0.03
3 weeks of recovery	**ORC3+3**	9/4	0.10 ± 0.09	0.08 ± 0.01	9/4	0.12 ± 0.02	0.14 ± 0.01
	ORC5+3	12/4	0.08 ± 0.02*	0.12 ± 0.01	9/2	0.15 ± 0.03	0.19 ± 0.05
	ORC9+3	13/5	0.11 ± 0.02*	0.14 ± 0.02	13/5	0.18 ± 0.04	0.22 ± 0.04
8 weeks of recovery	**ORC3+8**	7/5	0.12 ± 0.02	0.12 ± 0.02	7/2	0.21 ± 0.03	0.24 ± 0.01
	ORC5+8	10/5	0.11 ± 0.03	0.14 ± 0.03	10/3	0.22 ± 0.06	0.25 ± 0.06
	ORC9+8	11/5	0.10 ± 0.01*	0.13 ± 0.05	11/4	0.19 ± 0.04	0.22 ± 0.02

Values are expressed as mean ± standard deviation, t Test, *$p<0.05$, n: number of animals in surgery group/number of animals in sham group.

Table 3: Incidences of seminiferous tubules per class of histological alterations of the germinal epithelium in rats submitted to cryptorchidism and orchiopexy.

			Classes[a] (%)							
	Groups	N	1		2		3		4	
			Surgery	Sham	Surgery	Sham	Surgery	Sham	Surgery	Sham
Cryptorchidism	CPT+3	9/5	0	97.80 ± 0.79	0.45 ± 0.15	2.06 ± 0.91	71.81 ± 17.63*	0.13 ± 0.30	28.13 ± 17.71*	0
	CPT+6	10/5	0	99.10 ± 0.84	6.32 ± 19.90	0.89 ± 0.84	74.88 ± 21.24*	0	18.79 ± 17.84*	0
	CPT+9	9/6	0	99.00 ± 0.94	0	0.99 ± 0.94	63.08 ± 23.85*	0	35.92 ± 23.85*	0
3 weeks of recovery	ORC3+3	6/4	5.25 ± 7.43	97.55 ± 2.24	24.19 ± 17.69*	2.44 ± 2.24	53.15 ± 22.01*	0	17.39 ± 28.14	0
	ORC5+3	12/4	9.05 ± 13.46	94.91 ± 2.42	25.50 ± 14.76*	4.37 ± 2.51	53.55 ± 19.60*	0.63 ± 0.91	11.87 ± 14.42	0.07 ± 0.17
	ORC9+3	9/5	5.08 ± 6.97	99.04 ± 0.71	26.30 ± 13.46 *	0.95 ± 0.71	59.97 ± 23.47*	0	8.63 ± 9.84	0
8 weeks of recovery	ORC3+8	6/5	80.50 ± 21.57*	95.84 ± 7.40	14.91 ± 18.87	4.15 ± 7.40	0.64 ± 21.57	0	3.93 ± 6.89	0
	ORC5+8	7/4	69.97 ± 26.51 *	99.66 ± 0.32	17.89 ± 14.15	0.33 ± 0.33	6.02 ± 9.64	0	6.11 ± 8.03	0
	ORC9+8	6/5	77.13 ± 25.90*	98.74 ± 0.74	11.78 ± 16.90	1.25 ± 0.73	6.33 ± 8.67	0	4.76 ± 7.03	0

Values are expressed as mean ± standard deviation, Fisher exact test, *$p<0.001$, n: number of animals in surgery group/number of animals in sham group. [a]Classes of histological germ cell type predominance in the germinative epithelium: tubules containing spermatid and sperm (Class 1); tubules with spermatid and sperm with vacuoles, apoptotic bodies and giant cells (Class 2); tubules with spermatocytes and spermatogonia and vacuoles, apoptotic bodies and giant cells (Class 3); and tubules with Sertoli cells only (Class 4).

10. FIGURES

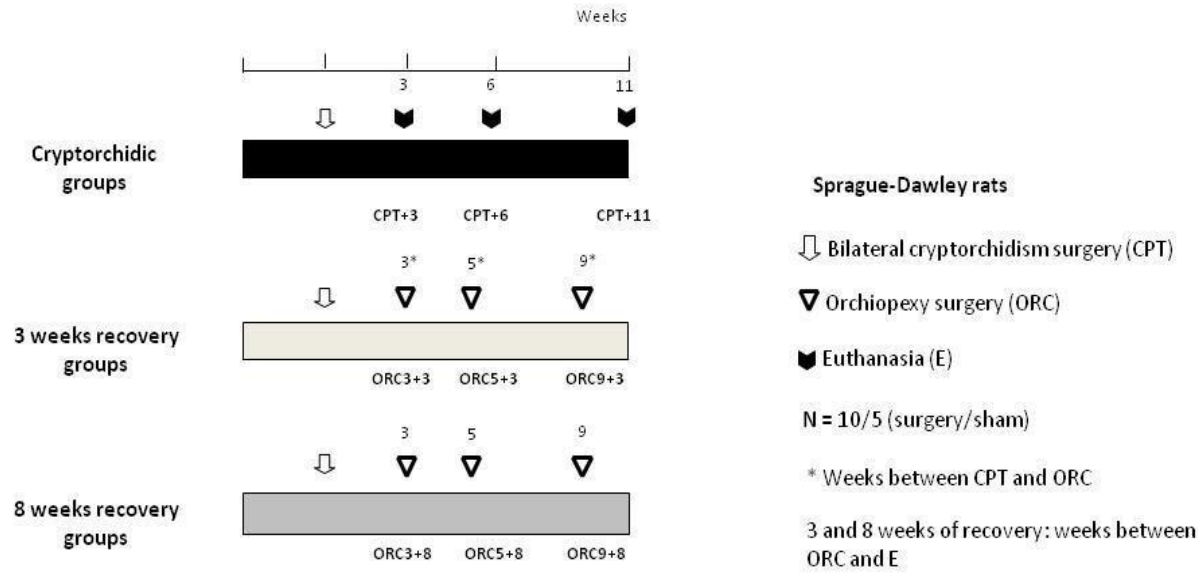

Figure 1: Experimental design.

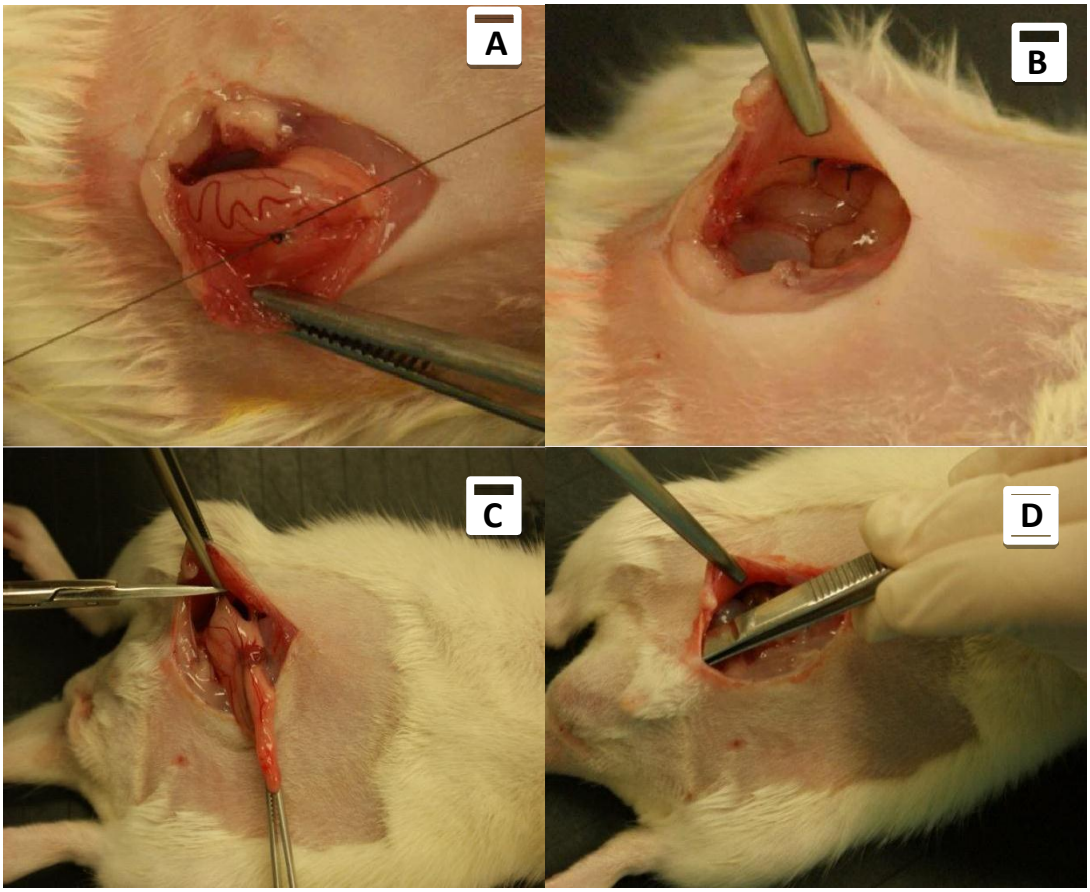

Figure 2: Surgical induction of cryptorchidism by anchoring the testis through the tunica albuginea and in the abdominal wall (A) using two stitches at the cranial and caudal gonadal regions (B). Orchiopexy surgery induced by cutting the sutures which held the testes in the abdominal wall (C) and guiding the testes attached to the muscular layer of the scrotum (D).

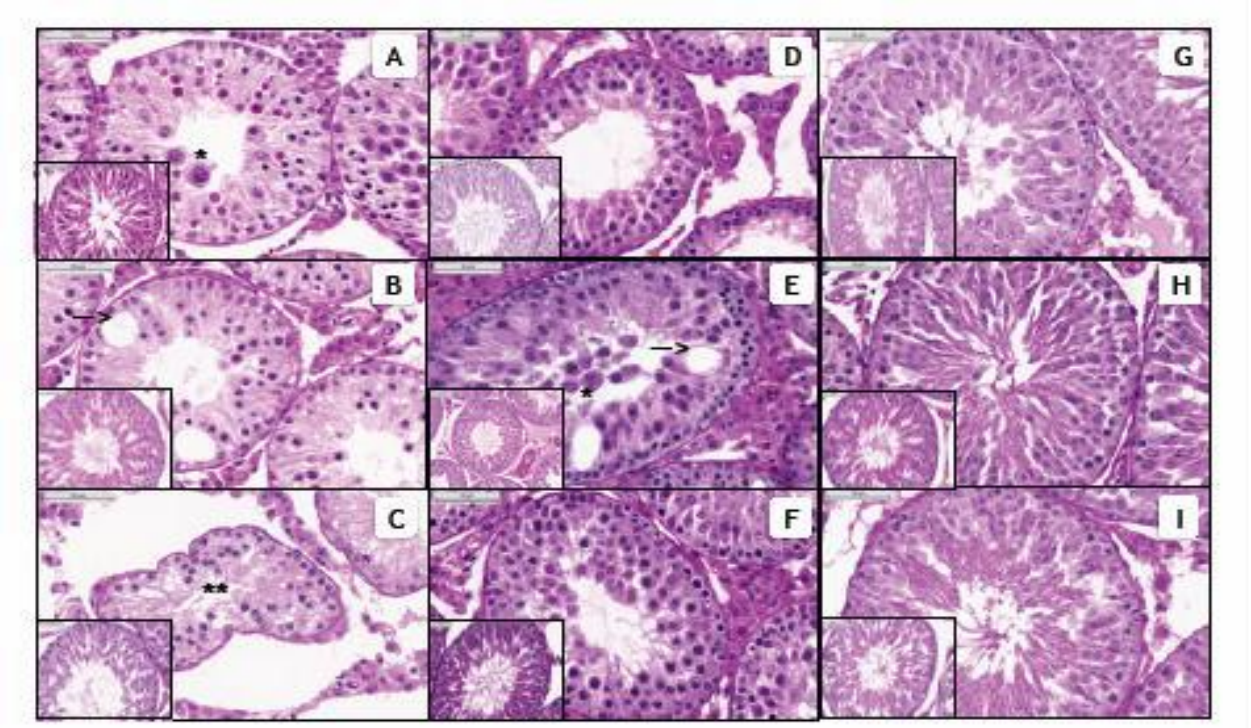

Figure 3: Histology of testicular alterations induced by cryptorchidism and testis recovery after orchiopexy. (A, CPT+3, B, CPT+6 and C, CPT+11) cryptorchidic testis showing germ cell lost, impairment of spermatogenesis, multinucleated cells (*), vacuoles (—>) and tubular atrophy (**). (D, ORC3+3, E, ORC5+3, and F, ORC9+3) After three weeks of orchiopexy, the spermatogenesis was partially recovered, with vacuoles still observed. (G, ORC3+8, H, ORC5+8 and I, ORC9+8) After eight weeks of orchiopexy, the germinative epithelium showed normal spermatogenesis.

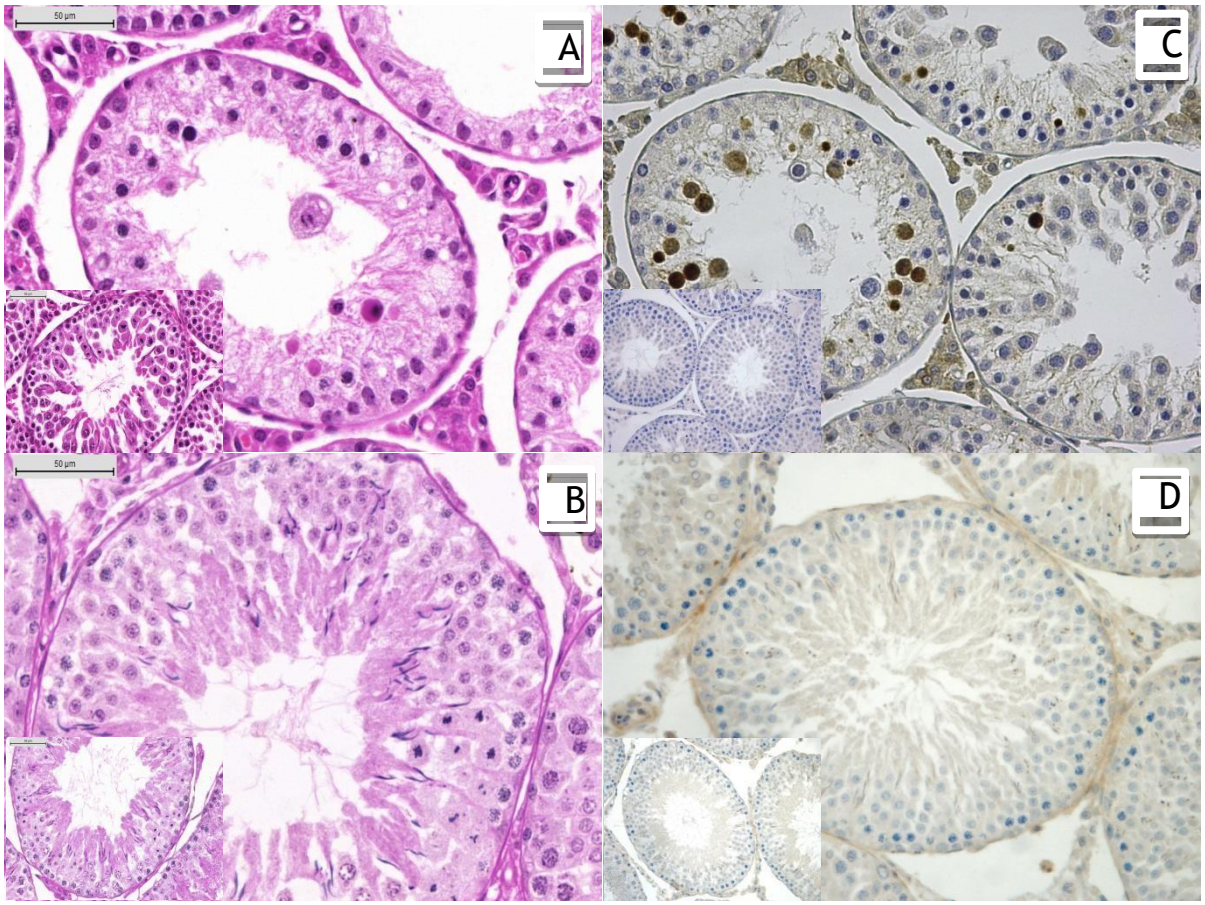

Figure 4: Mitosis and caspase-3 expression in rat testis. CPT+3 showing no mitosis (A) and high number of caspase-3 positive cells (C), 400X. ORC9+8 with cells undergoing division (B) and no caspase-3 expression (D), 400X. Insert for each figure shows the its respective sham.

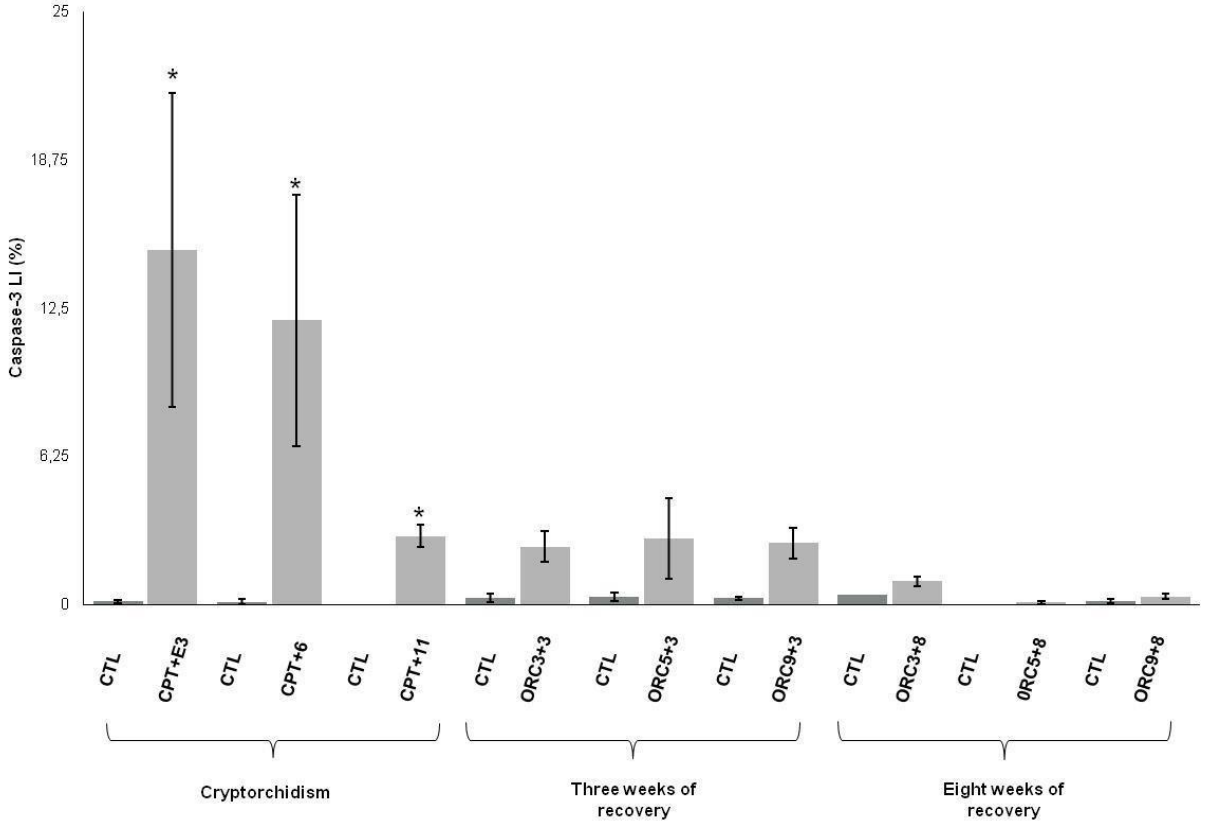

Figure 5: Caspase-3 LI (%). *p<0.05, Wilcoxon rank sum test, compared to their own sham groups.

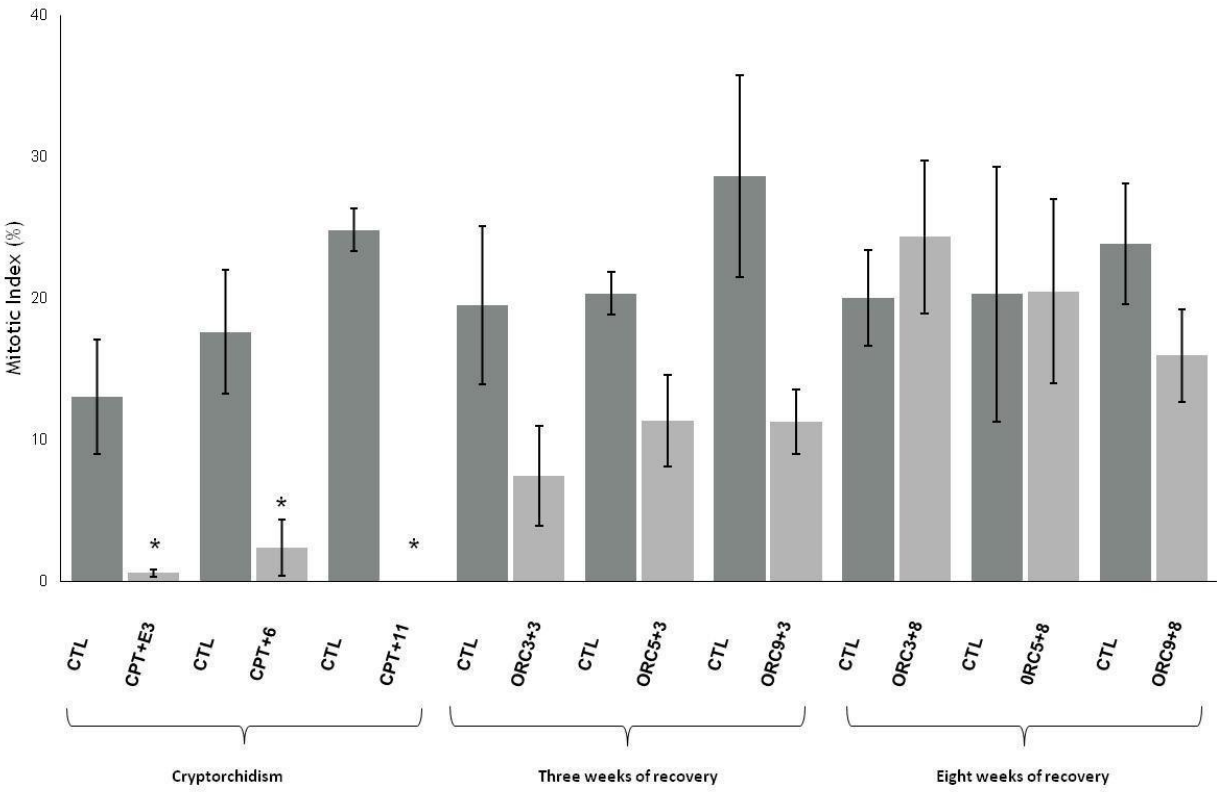

Figure 6: Mitotic Index (%). *p<0.05, Wilcoxon rank sum test, compared to their own sham groups.

Appendix

UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

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FMB

Ética CEEA
Comissão de Ética em Experimentação Animal

Criada através da Portaria DFM nº 30 de 26/04/99

Certificado

Certificamos que o (Protocolo CEEA 926/2012) Estabelecimento de modelo de tumores de células germinativas em testículos de ratos Sprague-Dawley, a ser conduzido por Merielen Garcia Nascimento, orientada pelo Prof. Dr. João Lauro Vianna de Camargo, com a colaboração de Ana Paula Ferragut Cardoso, Deilson Elgui de Oliveira, Samuel Cohen, Sohei Kitazawa e Wilma de Grava Kempinas, com o apoio técnico de Paulo César Georgete e Paulo Roberto Cardoso, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), com a ressalva de que os "ratos" são provenientes de Biotério convencional, sem condições de atestar a Sanidade dos mesmos.

Projeto de Pesquisa aprovado em reunião da CEEA em 29/03/2012.

Profª Drª Maria Rosa Bet Moraes Silva
Presidente da CEEA

Alberto Santos Capelluppi
Secretário da CEEA

Distrito Rubião Junior, s/nº - Botucatu – S.P. CEP: 18.618-970 Fone/Fax: (0xx14) 3811-6143 e-mail secretaria: capelluppi@fmb.unesp.br

Universidade Estadual de Campinas - UNICAMP
Centro Multidisciplinar para Investigação Biológica na
Área da Ciência em Animais de Laboratório – CEMIB
International Council For Laboratory Animal Science
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Prof. João Lauro Viana de Camargo

CPF 513.355.738-87

Distrito Rubião Jr, s/n - RUBIÃO JUNIOR

BOTUCATU - SP

Atestado de Saúde Animal

Atestamos que os Ratos (30 machos e 50 fêmeas) da linhagem **NTacUnib:SD,** provenientes da Divisão de Produção de Animais S.P.F. (Specific Pathogen Free) deste Centro, pertencem à categoria sanitária S.P.F. e apresentam-se isentos dos agentes patogênicos pesquisados pelo laboratório de controle de qualidade sanitária. Informamos que os mesmos encontram-se livres de outros agentes infecciosos capazes de causarem riscos à saúde humana. A validade deste Atestado consta da data dos últimos testes do programa de monitorização sanitária, rotineiramente realizados pelo Laboratório de Controle de Qualidade Animal - C.Q.S.(*).

Observação - O estado sanitário dos animais retirados do CEMIB nesta data será mantido se os mesmos forem acondicionados em equipamento adequado e o mesmo não for violado durante o transporte. A Instituição receptora deverá oferecer infra-estrutura e condições adequadas para a manutenção de animais da Categoria Sanitária livres de agentes patogênicos especificados (S.P.F.), alojando os animais em equipamentos e/ou salas dotadas de sistema de barreiras de proteção sanitária. Torna-se necessário manejo correto e a esterilização de todo material utilizado na rotina como: ração, maravalha/cama, bebedouros, água, gaiolas, tampas, e outros.

(*) Data dos últimos testes de monitorização sanitária realizados em Abril de 2012.

Campinas, 04 de junho de 2012.

Drª. Sônia Cano Montebelo Rachel
CRMV-SP: 4462 - Médica Veterinária
E-mail : soniacmr@cemib.unicamp.br

Prof.Dr.Rovilson Gilioli
Diretor do Centro Multidisciplinar para
Investigação Biológica
CEMIB/UNICAMP
E-mail: diretoria@cemib.unicamp.br

Rua Cinco de Junho, 230 – Cidade Universitária Zeferino Vaz – Barão Geraldo – CEP: 13.083-970 – Campinas - SP.
Fone/Fax: (19) 3521-7652 - Universidade Estadual de Campinas – CNPJ 46.068.425/0001-33