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Influence of open testicular biopsy in prepubertal rats on rats' adulthood fertility with correlation to serum levels of inhibin B and follicle stimulating hormone

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Abstract *Objective:* Open testicular biopsy (OTB) is one of the options to accurately assess fertility potential of the undescended testis. The aim of the study was to investigate consequences of OTB in prepubertal rats on their adulthood fertility.
Methods: Thirty-eight prepubertal male rats were divided into three groups depending on day 20 procedure. The first group was the control group, the second sham operated and the third has left OTB. Bilateral orchiectomy was performed on day 70 to all groups, with determination of serum inhibin B and follicle stimulating hormone (FSH). Removed testes were compared according to the weight, volume, spermatogenesis, histological and apoptotic changes in both testes with differences in serum levels of inhibin B and FSH.
Results: Ipsilateral testicular weight, volume, and spermatogenesis reduction with a reduction of tubular number, diameter and germinative epithelium was found in OTB group. Significant increase in apoptotic index was found in biopsied testis without compensatory hypertrophy of contralateral testis. Differences of inhibin B and FSH were not statistically significant among three groups.

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Conclusion: OTB in prepubertal rats has detrimental effects on fertility in adulthood. It does not cause compensatory hypertrophy of the contralateral testis nor does it disturb serum levels of inhibin B and FSH.

Introduction

Male sterility can be considered as the main complication of cryptorchidism. Although semen analysis is routinely used to evaluate male infertility, sperm measurements that discriminate between fertile and infertile men are not well defined. According to Guzick et al. [1], subfertile ranges for men are those with sperm concentration of less than 13.5×10^6/mL, less than 32% of sperm with motility, and less than 9% with normal morphologic features. Fertile ranges are those with a concentration of more than 48.0×10^6/mL, greater than 63% motility, and greater than 12% normal morphologic features. Values between these ranges indicate indeterminate fertility. Despite these threshold values, none of them, alone or in combination, can be considered diagnostic of male infertility, although percent of normal morphology was the most significant predictor of pregnancy [1].

As a result of problems with infertility associated with cryptorchidism, it has for many years been treated either surgically or conservatively to descend retained testis into the scrotum and thereby reduce infertility rate. Despite current treatment options, fertility failed to improve in all cryptorchid patients even after successful therapy. Minipuberty, which occurs from 2 to 3 months of age, is very important for the preservation of male fertility [2]. It represents the sudden increase in gonadotropin secretion which leads to conversion of gonocytes to Adult dark (Ad) spermatogonia, from which the spermatocytes and sperm cells evolve. It does not happen in cryptorchid testes, but there is a reduction in the total number of gonocytes. After the sixth month of life, the reduction process leads to a significant reduction in total germ cell number at the end of the child's first year of life [2]. Current thinking recommends early surgery for cryptorchidism until the age of 1 year, to prevent deterioration in number of gonocytes [3]. Additionally, transformation of gonocytes into Ad spermatogonia together with orchidopexy is emphasized as the cornerstone to preserve fertility in cryptorchidism [4]. Before or after orchidopexy, hormonal therapy with gonadotropin-releasing hormone (GnRH) and human chorionic gonadotropin (hCG) is advised to artificially stimulate minipuberty [5]. The only way to accurately prove the success of hormone therapy or to evaluate testicular status after which hormone therapy may be started if it is indicated, is the testicular biopsy which may be performed during the orchidopexy. It is known that unilateral testicular injury may cause ipsilateral atrophy and damage to the contralateral testis, with decreased fertility and reduction in serum levels of inhibin B whose low serum levels may reflect damage to the contralateral testis [6]. As testicular maturation is not complete until puberty, it is expected that testicular injury before puberty will probably affect spermatogenesis in adulthood [7].

The aim of this study was to determine the influence of open testicular biopsy in prepubertal rats to the damage of ipsilateral and contralateral healthy testis, to fertility reduction in their adulthood, and to serum levels of inhibin B and FSH.

Materials and methodology

Thirty-eight Sprague—Dawley male rats were used as the experimental model ($N = 38$). The number of animals was determined in accordance with the data available in the literature for the serum levels of inhibin B and apoptotic index [8]. Accordingly, expected value of inhibin B after puberty in the control group of rats was 427 ± 20 pg/mL, and 280 ± 30 pg/mL in the biopsied group. For a significant difference of $p = 0.05$ and a 90% of test power, the expected test groups should contain the three rats. In compliance with the study of Wang et al., at least a four times increase of apoptotic index was expected in our experimental group of rats after the biopsy on day 70 of life. To prove a statistically significant change of apoptotic index four times increase in the biopsied group of rats compared with the control group at the significance level $\alpha = 0.05$ and for the test strength of 90%, 11 rats were required per group [9]. To perform this experiment, approval was obtained from the ethical committee of Split University Hospital Centre. At the age of 20 days (average age 20.9 ± 0.83) animals were randomly divided into three groups (12 rats in the first two groups and 14 rats in the third group). All animals were anesthetized using intraperitoneal urethane injection (20% solution, 10 mL/kg). After anesthesia, blood was taken from the femoral vein to determine serum levels of inhibin B and FSH. Group I was the control and the only blood was taken on day 20 of life. Group II was sham operated on day 20, and, after the blood was taken, left testis was approached, testis volumes were measured, and scrotal wall was closed with the resorptive sutures polyglactin 910 5/0 (Vicryl, Ethicon, Johnson & Johnson Somerville, NJ, USA) followed by application of povidone iodine.

In Group III the blood samples were taken on day 20 of life. Left testes were approached, testis volumes were measured, and an open testicular biopsy was performed. Tunica albuginea was pierced with a scalpel in the upper testicular pole to obtain a testicular tissue sample of size $2.86 \pm 0.67\ mm^3$. The biopsy sample volumes were measured and calculated from their histological slices interpolating the longitudinal and transverse length of slices, presenting approximately 1.67% of testicular volumes. The tissue samples were fixed in 4% buffered formaldehyde for histological analysis. Defects in tunica albuginea and scrotal skin were

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closed with resorptive sutures polyglactin 910 5/0 (Vicryl), and the wound was dressed with povidone iodine. On day 70 of life (average age 69.7 ± 1.5) all rats underwent bilateral orchiectomy under general anesthesia and blood samples were taken to determine serum levels of inhibin B and FSH. Weight, height, length, and width of the removed testes were measured, and testicular volume was calculated using formula: length × height × width × 0.71 [10].

Epididymal tail and the initial part of the ductus deferens were removed and stored in collagenase to obtain sperm from which number, motility, and morphology of sperm cells were determined. The remaining part of the epididymis and the testis were fixed in 4% formaldehyde for histological and immunohistochemical analysis. Upon completion of the experiment the animals were sacrificed.

The tissue was processed using standard procedures of dehydration and waxing in histokinet Thermo Shandon. Preparations were embedded in paraffin and cut into 5 μm slices, mounted on examination glasses and fixed in thermostat for 15 min at 58 °C. Preparations were then deparafinized, dehydrated and stained in automatic stainer (Sakura, Tokyo, Japan).

Representative paraffin blocks of all cases were elected for immunohistochemistry in which the histological preparations were cut and mounted on silanized slides. Slides were stained in an automatic machine immunohistochemical stainer Ventana Benchmark. Incubation with primary rat antibody on rat cleaved caspase 3 (Asp 175 cleaved caspase 3, Cell Signaling, USA) was 60 min. After the application of the primary rabbit antibody to rat cleaved caspase 3, apoptotic cells painted intensively diffuse or granularly with cytoplasmatic paint. Total number of cells was counted on five randomly selected fields on a light microscope under a large increase and Image analysis computer program (Soft Imaging System GmbH, Munster, Germany) and the expression was determined quantitatively, counting positive cells per 100 cells. The results have been shown as an apoptotic index, which represents the number of positively stained cells to hundreds of cells in the visual field of light microscope (the number of positive cells/100 cells).

Serum levels of inhibin B were measured using ELISA (Rat inhibin B, INH-B ELISA Kit, Cusabio, China). Serum FSH levels were determined using ELISA (Rat follicle-stimulating hormone, FSH ELISA Kit, Cusabio).

Samples of epididymal tissue to analyze the number, mobility, and structure of the sperm cells were stored in collagenase and incubated for 90 min at 37 °C in a CO_2 incubator. Then the tissue was removed from the incubator and abundantly macerated using sterile needles, after which it was centrifuged at 500–800 g/10 min. Supernatant with collagenase was removed and resuspended in 0.5 mL of medium for sperm flushing after which 10 μL of the suspension was placed in the chamber for sperm cell counting (Makler counting chamber, Sefi-Medical Instruments, POB 7295, Haifa 31070, Israel).

Obtained data were analyzed using Microsoft Excel for Windows Version 11.0 (Microsoft Corporation, USA) and Statistica for Windows Release 12.0 (Statsoft Inc., Tulsa, OK, USA). All p values of less than 0.05 were considered to indicate statistical significance.

Results

Statistically significant difference was found in the weight of the left testes in relation to the intervention ($p = 0.018$). At the time of the first surgery there was no statistically significant difference in the volume of the left testes between groups II and III ($p = 0.360$). There was a statistically significant difference of left testicular volume in relation to the intervention ($p < 0.001$). There was no statistically significant difference between the volume of the right testes on day 70 of life between the groups ($p = 0.254$). Statistically significant difference was found in weight ($p = 0.015$) and volume ($p < 0.001$) ratio between the left and right testes in relation to the intervention. Volume ratio varied by 15% between groups II and I ($p = 0.007$). In group III volume ratio was 66% lower than in group I ($p < 0.001$) and 50% lower than in group II ($p = 0.003$) (Table 1).

Between the left epididymal sperm counts in relation to intervention statistically significant difference was found ($p < 0.001$). Sperm count in group III was lower by 11 million compared with group I ($p = 0.001$), and 10.5 million compared with group II ($p < 0.001$).

Table 1 Arithmetic mean of testicular volume 20th day and testicular weight and volume 70th day, with the medians of left/right testicles weight and volume 70th day.

	Group I ($n = 12$)	Group II ($n = 12$)	Group III ($n = 14$)	p value
Left testicles weight 70th day (g)	3.7 ± 0.56	3.4 ± 0.71	2.4 ± 1.3	0.018[a]
Right testicles weight 70th day (g)	3.8 ± 0.52	3.6 ± 0.3	3.5 ± 0.83	0.875[a]
Weight ratio of the left and right testicles 70th day (%)	97 (83–115)	100 (53–110)	67 (18–174)	0.015[b]
Volume of the left testicles 20th day (cm^3)		0.155 ± 0.036	0.17 ± 0.068	0.360[c]
Volume of the left testicles 70th day (cm^3)	2.25 ± 0.598	2 ± 0.95	1.03 ± 0.68	<0.001[a]
Volume of the right testicles 70th day (cm^3)	1.94 ± 0.31	2.29 ± 0.77	2.1 ± 0.48	0.254[a]
The volume ratio of the left and right testicles 70th day (%)	110.5 (83–219)	94.9 (25.8–124)	44.5 (63–131.6)	<0.001[b]

[a] ANOVA.
[b] Kruskal–Wallis test.
[c] Student t-test.

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Comparing only the left epididymises with sperm, statistically significant difference was found between the percentage of mobile sperm cells in relation to intervention ($p = 0.028$). Percentage of mobile sperm cells in group III was lower by 20.8% compared with group I ($p = 0.009$), and 21.4% lower than in group II ($p = 0.021$).

Also, statistically significant difference was found between percentage of non-mobile sperm cells on day 70 of life in the left epididymis in relation to the intervention ($p = 0.028$). Percentage of non-mobile spermatozoa in group III increased by 21% compared with group I ($p = 0.009$), and was 21% higher than in group II ($p = 0.021$). In the number and percentage of sperm cells in the right epididymises, statistically significant difference was not found between the groups (Table 2; Fig. 1a).

Regarding tubular diameter of the left testes on day 70 of life in relation to the intervention, statistically significant difference was found between the groups ($p = 0.001$). A statistically significant difference was found in the germinative epithelium thickness of the left testes in relation to the intervention ($p < 0.001$). In group III germinative epithelium thickness was 61.5 μm lower than in group I ($p < 0.001$) and 60 μm lower than in group II ($p = 0.005$). There was a statistically significant difference between the values of the median Johnsen score in left testes in relation to the intervention ($p < 0.001$). In group III median Johnsen score was lower for the seven compared with group I ($p < 0.001$) and group II ($p < 0.001$) (Table 2; Fig. 1b). It is noteworthy that no statistically significant difference was found in the right testis on day 70 in the tubular diameter, thickness of germinative epithelium, or median Johnsen score.

There were no interstitial widening and inflammatory changes in the left testes in the first and second group of rats, whereas the third group of rats had widened testicular interstitium in 10 of 14 testes ($p < 0.001$), 12 of 14 testes had marked inflammatory process ($p < 0.001$) (Fig. 1c). Diffuse left testes atrophy occurred in 13 of total 14 testes in group III, whereas atrophy occurred in only two testes of group I and II ($p < 0.001$). The right testes of all groups of rats did not reveal any signs of atrophy, inflammation, nor widening of the interstitium.

Apoptotic index was 0 in the left and right testes in groups I and II on day 70 of life, whereas the median apoptotic index in the left testes of group III was 3 (0–11) (Fig. 1d), and it was 0 (0–1) in the right, nonbiopsed testes of the same group.

Statistically significant difference in inhibin B ($p = 0.482$) and FSH values ($p = 0.882$) between groups on days 20 and 70 was not found (Table 3).

Discussion

After recent findings about germ cell physiology and new opportunities for the treatment of undescended testes, attention is drawn to testicular biopsy in prepubertal boys once again. Although testicular biopsy plays an important role in treatment of adult male infertility, it is currently the only method that can be used to: a) predict future fertility, b) assess success of initial treatment, and c) set up indication for possible further treatment [11]. For these reasons, and because of doubts about the harmful effects of biopsy on testis, the subject of this study was to ascertain possible harmful effects to the biopted and opposite nonbiopted testis and the possible impact on future fertility in adulthood.

Literature review revealed numerous studies that focused on association of testicular trauma with subsequent infertility [7,12–15]. Different studies showed a decrease in fertility after blunt testicular trauma [13–17]. Nolten et al. reported high incidence of remote blunt testicular trauma with hormonal changes of FSH along with semen abnormalities [18]. Slavis et al. found that trauma to the rat testis produced contralateral changes [13]. Contrary to the above findings, Shaul et al. reported no changes in contralateral Johnsen testicular maturation score [17]. The present study showed expectedly lower weight and volume of biopsied testes than were noted in the contralateral nonbiopsied testes and testes in group I and II. Furthermore, semen analysis showed impairment of spermatogenesis in biopsied testes while there were no changes in spermatogenesis in contralateral testes of same group and in the first two groups. Histologically, the main changes observed after

Table 2 Median of sperm cell number and the percentage of mobile, non-mobile, and abnormal sperm cells in left and right epididymis with median Johnsen scores on 70th day of life.

Sperm cells 70th day		Group I ($n = 12$)	Group II ($n = 12$)	Group III ($n = 14$)	p value
Left	Number (mil.)	11 (5–21)	10.5 (0–18)	0 (0–9)	<0.001
	Mobile (%)	36.4 (20–60)	37 (3.8–50)[a]	15.6 (0–25)[a]	0.028
	Non-mobile (%)	63 (40–80)	63 (50–96)[a]	84 (75–100)[a]	0.028
	Abnormal (%)	31 (20–66)	32.5 (20–64)[a]	32 (0–38)[a]	0.805
	MJS (1–10)	10	10 (3–10)	3 (2–9)	<0.001
Right	Number (mil.)	10 (4.7–16)	14 (8–18)	11 (6–19)	0.115
	Mobile %	31.6 (10.1–58.3)	39.8 (12.5–50)	33.3 (1.6–52.6)	0.359
	Non-mobile %	68.3 (41.6–89.9)	60.2 (50–87.5)	66.7 (47.4–98.3)	0.359
	Abnormal %	34 (24–64)	33 (22–66)	30 (22–67)	0.489
	MJS (1–10)	10	10	10	

Kruskal–Wallis.
MJS = Median Johnsen score.
[a] Percentages of mobile, non-mobile, and abnormal sperm cells were calculated only in relation to the number of left epididymises in which there were sperm cells $n = 10$ in group II, and $n = 4$ in group III, without aspermicepididymises.

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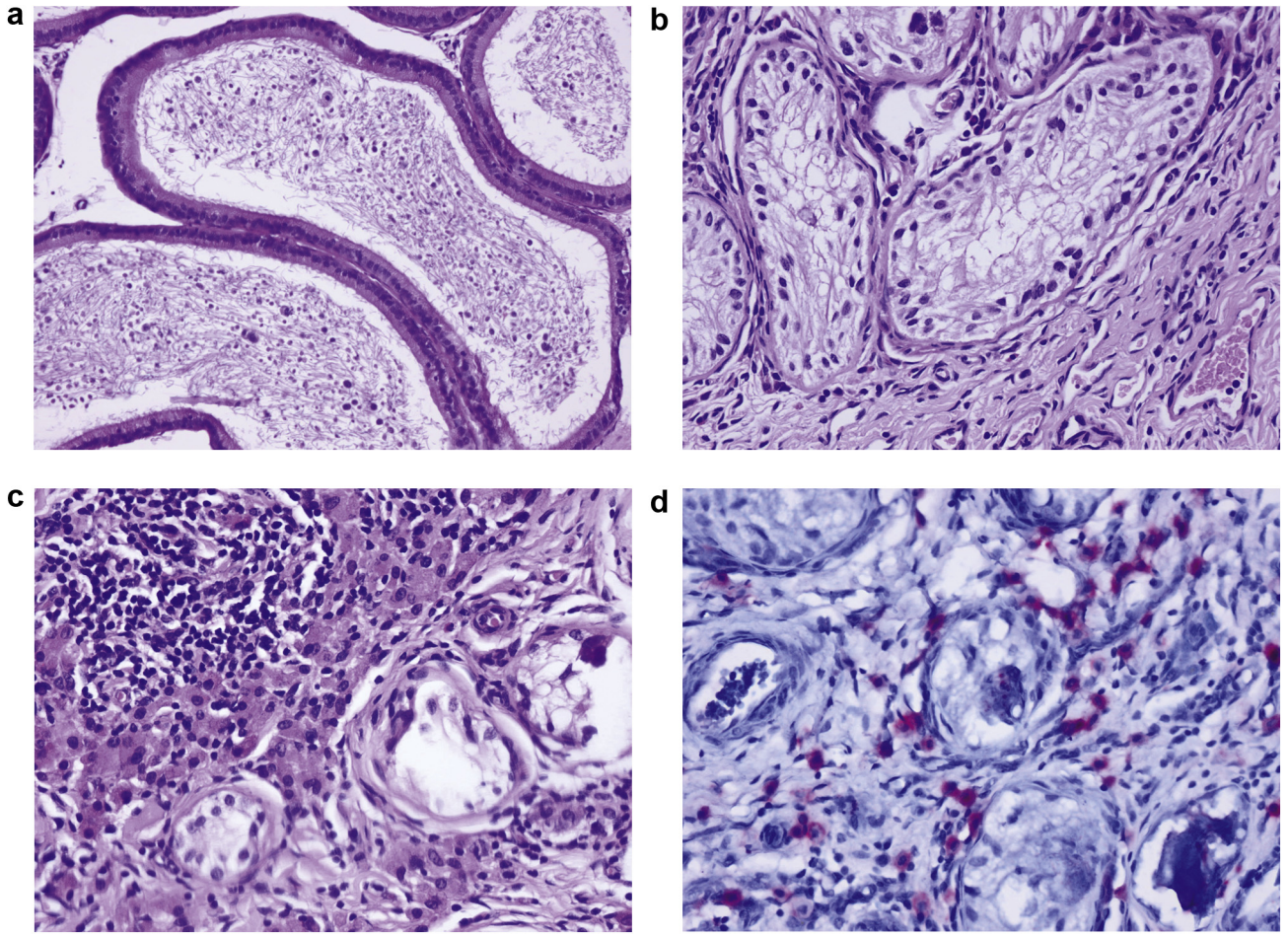

Figure 1 (a) Disturbed spermatogenesis with significant percent of dismorphed spermia in epididymal ducts (HE 400×). (b) Impaired spermatogenesis in empty seminal ducts with Sertoli cells on the basal membrane, Johnsen score 2 (HE 400×). (c) Inflammation with atrophic seminal ducts which are surrounded by fibrosing and inflamed interstitium (HE, 400×). (d) Apoptosis shown as red colored cells in peritubular interstitium and less in seminal ducts (cleaved caspase 3/AP400×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

biopsy were testicular inflammation, atrophy with reduction in the number and diameter of testicular tubules, and lowering in median Johnsen maturation score. No changes were observed in contralateral testes.

Normally, spermatozoa express antigens but are isolated from immune-system recognition by the blood-testis barrier consisting of the tight junctions of Sertoli cells [19]. Interruption of that defense mechanism would lead to an immunological response that could be antibody- or cell-mediated autoimmunity. Such a response was reported after testicular torsion [20] and experimental cryptorchidism [21]. In contrast, Wallace et al. reported changes in the contralateral testis with no evidence of a cell-mediated reaction after injury to one testis [22]. Recently, investigation into ischemic trauma to the testis found that mechanisms responsible for increased apoptosis in the ischemic testes include production of numerous cytokines, enzymes, and mediators of inflammation in ischemic testis

Table 3 Inhibin B and FSH values, 20th and 70th days of life and their changes.

	Day	Group I (n = 12)	Group II (n = 12)	Group III (n = 14)	p value
Inhibin B pg/mL	20	24.0 ± 10.1	18.2 ± 6.5	21.0 ± 5.8	
	70	61.5 ± 38.2	85.8 ± 63.7	83.5 ± 54.9	
Change		27.5 (−29 to 128)	67 (−16 to 179)	43.8 (−9 to 216)	0.482
FSH ng/mL	20	11.6 ± 2.7	10.8 ± 4.0	10.8 ± 3.8	
	70	11.6 ± 4.1	13.1 ± 1.8	11.6 ± 3.8	
Change		0.35 (−6.5 to 6.2)	1.2 (−6.8 to 6.7)	0.58 (−4.9 to 6.5)	0.882

Kruskal–Wallis.

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rather than overproduction of oxygen free radicals or other products of reperfusion, which also may induce programmed cell death in the contralateral testis [23—25]. The measurement of apoptosis in the contralateral testis demonstrated an increase in germ cell programmed death that may cause loss of fertility, although there are studies which show that testicular ischemia to one testis produces minimal contralateral damage [25,26]. In contrast to germ cell apoptosis, endocrine profile seems to be more resistant to ischemia [27].

In this study apoptotic index was compared on day 70 of life in biopsied and contralateral testis. Apoptotic index was not statistically significant between the groups, but apoptosis was present in some of the contralateral testes in group III. Such a small number of apoptotic positive cells, despite significant histological changes in the biopsied testes, is probably caused by the bulk of apoptosis occurring shortly after the testicular biopsies.

It has been well documented that unilateral castration of sexually immature animals results in hypertrophy of the contralateral testis [28]. Guarino et al. found that the evaluation of testicular volume is not predictive for testicular dysfunction in adolescent boys [29]. Despite increased testicular volume, compensatory testicular hypertrophy does not seem to improve semen quality [28]. Our results indicate significantly worse damage to biopsied testis compared with the contralateral testis. Although testicular biopsy resulted in significant damage to the biopsied testes, compensatory hypertrophy was not observed in contralateral testes as it was reported by Stampfli and Hadziselimovic [30]. Several studies examined hormonal aspects of compensatory testicular hypertrophy in prepubertal rats and have reported increased levels of FSH [31]. FSH and other factors produced by germ cells are regulators in inhibin B production, which is a glycoprotein produced by Sertoli cells [32]. Recently, inhibin B was described as a marker of Sertoli cell function, with high sensitivity in predicting quality of spermatogenesis in adults [33]. Carillo et al. reported normal values of inhibin B in nine boys with either unilateral or bilateral varicocele [34]. Romeo et al. showed reduction of inhibin B in boys with varicocele and early arrest of spermatogenesis with a significant positive correlation between inhibin B and testicular volume [35]. Significant variations of inhibin B and FSH between groups according to open testicular biopsy were not found in our study, probably because in all groups of rats Johnsen testicular maturation score was greater than 2, which confirms the findings of Sertoli cells in tubules that secrete inhibin B and did not lead to consequential compensatory hypertrophy of the contralateral testis.

Conclusion

Open testicular biopsy in prepubertal rats is technically demanding to execute. Despite usage of fine microsurgical technique during procedure, a harmful effect was found on fertility to the ipsilateral testis in adulthood, but without damage to the contralateral testis. This means that bilateral testicular biopsy would cause infertility in the adult rat. However, open testicular biopsy in prepubertal rats is not a harmless procedure in itself and should therefore be used with great caution during the cryptorchidism procedure, especially taking into account possible primarily damaged testes.

Conflict of interest

None.

Funding

None.

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