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ORIGINAL ARTICLE

Effects of laparoscopic division of spermatic vessels on histological changes of testes: long-term observation in the model of prepubertal rat

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Abstract Laparoscopic Fowler-Stephens and Palomo procedures are now commonly performed in children with high positioned intra-abdominal cryptorchidism and varicocele, respectively. During the procedures, the spermatic vessels are ligated and therefore the question of risk related to testicular atrophy is often raised. The long-term follow-up of the histology after the procedures is rare. In this study, we simulated a laparoscopic spermatic vessels clipping and division (SVCD) in a prepubertal rat model, and examined the histological alterations of the testes with regard to spermatogenic arrest between prepuberty and middle age. Thirty-day-old Wistar rats divided randomly into three groups underwent laparoscopic sham operation, unilateral SVCD and unilateral SVCD with additional contralateral orchiectomy, respectively. Histological investigations observed on semithin and paraffin sections were performed at seven different postoperative intervals between day 9 and day 540. We defined partial, most and complete spermatogenic arrest of the seminiferous tubules to correspond with mild, severe spermatogenic arrest and atrophy, respectively. Laparoscopic SVCD induced testicular spermatogenic arrest in a total of 85% of the operated testes with different severity; 27% of operated testes with mild or severe spermatogenic arrest were seen between puberty and middle age (day 45–540 postoperative), and their size was only slightly reduced. Of the operated testes, 51% showed atrophic signs with a striking decrease in size, and their contralateral testes revealed in all cases mild or severe spermatogenic arrest started as early as day 45 postoperatively. Parallel to the spermatogenic arrest, Leydig cell hyperplasia developed frequently in impaired testes, especially in those without contralateral testes, finally reaching a typical adenoma size. Laparoscopic SVCD in prepubertal rats could disturb spermatogenesis with differing severity in most cases. This impairment could persist from peripuberty to middle age, and even involve the contralateral testes, in the case of operated testes and show complete spermatogenic arrest. This study showed that laparoscopic SVCD may have high risk in compromising the operated testis.

Keywords Testis · Spermatic vessel · Division · Spermatogenesis · Laparoscopy

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Introduction

Currently, laparoscopic Fowler-Stephens and Palomo procedures are common approaches for children with high positioned cryptorchidism and varicocele [1, 2], respectively. During the procedures, whole spermatic vessels are ligated and divided [3]. The survival of the testis depends on its collateral circulation provided by the vessels of vas deferens and cremaster. So, the procedure raises often the question of risk of insufficient collateral blood supply, which may lead to testicular atrophy. Clinically, ligation of spermatic vessels by open surgery was believed to produce

testicular atrophy in 20–50% of cases [4]. Multi-institutional analysis subsequent to laparoscopic Fowler-Stephens procedures showed an atrophic incidence of 14.1% [5]. However, to our knowledge, the term "testicular atrophy" was used without any standard definition and was often solely based on the evaluation of the changes in testicular size or volume [6]. On the other hand, children rarely have follow-up examination after the operation into adulthood because different institutions and different physicians are involved.

The rat is a commonly used animal model for cryptorchidism and varicocele research [7, 8]. Its testicular descent starts on the 10th postnatal day and is complete at around 3 weeks. At this time, spermatogonia and spermatocytes occupy the seminiferous tubules (ST). Round spermatides, which have a PAS-positive acrosomal granule, appear in the 4-week-old testis. The 10-week-old testis shows a completely mature appearance, containing spermatogonia and all the differentiating cells from spermatides up to spermatozoa (Fig. 1; [9]). So, the 30-day-old rat was often used as a prepubertal model for research on the spermatic vessels ligation of high undescended testis [10, 11].

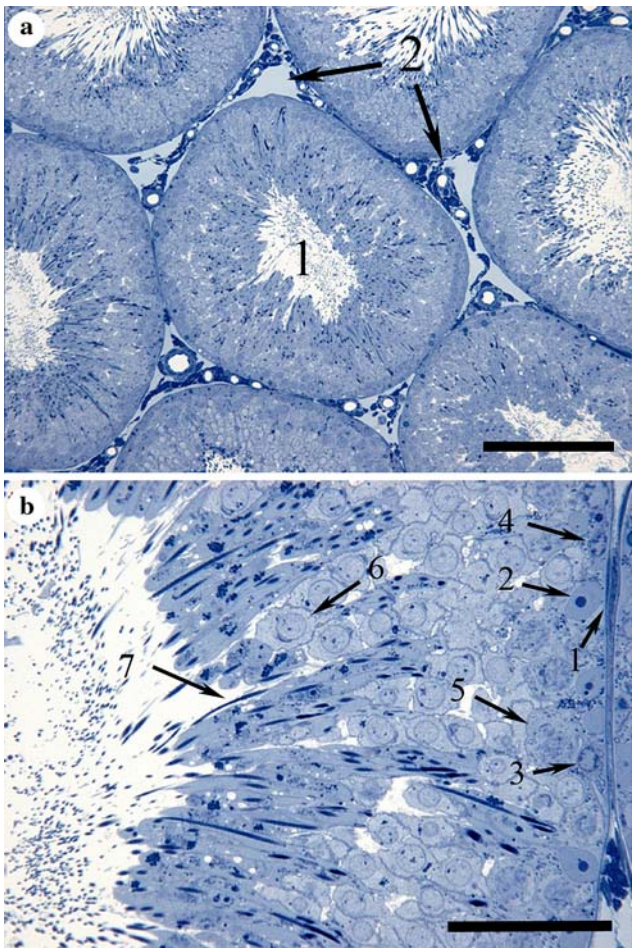

Fig. 1 **a** Normal seminiferous tubule and interstitial space (4-month of age). *1* Seminiferous tubule. *2* Interstitial spaces filled with Leydig cells. Bar = 150 μm. **b** Normal seminiferous epithelium with full spermatogenesis (4-month of age). *1* Basal lamina. *2* Sertoli cell nucleus. *3* Type A spermatogonium. *4* Type B Spermatogonium. *5* Spermatocyte. *6* Round spermatide. *7* Immature spermatozoa. Bar = 30 μm

Histological studies after simulated ligation of spermatic vessels in rats have been carried out [8, 12]. However, these studies involved the prepubertal stage, and observation from prepuberty to middle age is rare. Furthermore, studies with tissue perfusion fixation and observation of histology on semithin sections, which can provide more informative alteration of histology than on paraffin sections [13], are lacking. There is no study that simulates spermatic vessels clipping and division (SVCD) with laparoscopy, which imitates the clinical procedure and minimally disturb the sensitive spermatogenesis of the rats. Hence, the present study simulated the laparoscopic SVCD in prepubertal rats and determined whether this procedure has a high risk of compromised spermatogenesis, in the involved testes, which may persist to peripuberty or even middle age.

Materials and methods

Thirty-day-old male Wistar rats (Boehringer Ingelheim Pharma KG, Germany) were housed in metal cages and maintained in a room with controlled illumination (14 h light: 10 h darkness, lights on at 0500 hours), and temperature (25°C) with ad libitum access to commercial pellets and tap water. Care was provided in accordance with the guidelines published in the 1984 Guide for Care and Use of Laboratory Animals (National Institutes of Health, publication no. 85–23, 1985).

All rats were anesthetised under ether inhalation followed by intra-peritoneal injection of thiopental sodium. A 3 mm transverse abdominal incision was made in the one-third upper portion of the xiphoid-pubic line. After insertion of a 2 mm diameter telescope (0°, 7210 AWA, Karl Storz Endoscope, Germany) into the peritoneal cavity, CO_2 pneumoperitoneum was set up by a plastic cannula (22G) that penetrated the abdominal wall. A 1.7 mm port was placed into the abdominal cavity. The following procedures were performed according to the experimental design. The rats were randomised into three groups: group I underwent sham operation as control; group II underwent unilateral SVCD; group III underwent unilateral SVCD plus contralateral orchiectomy. After the operation, the rats of seven subgroups (six in each) in all three groups were killed on day 9, 20, 45, 90, 180, 360 and 540, respectively.

At least two rats of each subgroup were fixed via perfusion of the abdominal aorta using 1.5% glutaraldehyde, 1.5% paraformaldehyde, 2.5% polyvinylpyrrolidone in

0.1 M PBS (pH 7.4) for 30 min. The organs were removed and 100 μm thick sections were prepared using a Dosaka-microslicer. After cytochemical staining, postfixation with 1–2% osmium tetroxide, the sections were embedded in Epon or Araldite, as described [14]. Serial semithin sections (0.5–1 μm) were stained with a modified Richardson solution (methylene blue-Azur II). The testes of the other rats were directly removed, immersed in the same fixative as mentioned previously and embedded in paraffin. Paraffin sections of 5 μm thickness were then stained with eosin and hematoxylin. In case of calcification, the testes were treated with EDTA for 24 h before embedding.

The length and width of the testes were measured and the testicular size was calculated. The maturation of STs was assessed applying the Johnson score with a scale of 1–10 [15]. Using NIH image software (U.S. National Institutes of Health, Springfield, VA, USA), qualitative and quantitative estimations were performed on histological sections obtained from semithin and paraffin sections. Images were taken using a digital camera (Leica DC300) mounted on a Leica microscope and captured with a PC using adobe Photoshop 7.0. The area of the STs was measured at five areas on 30 smallest and intact tubules by tracing the periphery at 50× magnification. $P < 0.05$ was considered to be statistically significant.

Results

The complete procedure was carried out within 6 min. No rat was lost during laparoscopy, but two died by ether inhalation before the operation commenced; 12 rats were lost during postoperative breeding, and 2 that developed spontaneous abdominal and skin tumours were excluded. A total of 185 testes were examined, 72 from 36 animals in group I, 78 from 39 animals in group II and 35 from 35 animals in group III.

Histology revealed large variations, ranging from normal appearance (Fig. 1) to complete spermatogenic arrest (atrophy). There was heterogeneous impairment of STs in the testes. However, three different patterns could be distinguished: (1) Mild spermatogenic arrest: evident dilation of the intertubular space; 20% or more of the STs showed expansion of the intercellular spaces in the adluminal or basal compartment, appearing as "vacuoles" preferentially located at the blood–testis barrier. The basal lumina surrounding atrophic STs was slightly thickened and less than 5% STs were lined exclusively by Sertoli cells. (2) Severe spermatogenic arrest: conspicuous enlargement of intertubular space and 50% or more STs exhibited evident reduction of seminiferous epithelium and expanded tubular lumen (Fig. 2a); More than 5% of STs showed the phenotype of Sertoli cell only, or imcomplete spermatogenesis. The basal lamina was markedly thickened and the myoid cell layers surrounding the STs were numerically increased (Fig. 2b). (3) Complete spermatogenic arrest (atrophy): loss of normal testicular architecture and massive necrosis of both compartments in the central portion of the testis, accompanied by early calcification and vascular remnants. Almost no germ cell survived. Of the 74 SVCD testes from group II and III, 11 (15%) presented normal appearance, 18 (24%) exhibited mild and 7 (10%) revealed severe spermatogenic arrest, and 38 (51%) showed atrophy at different postoperative intervals. The 13 mild and all 7 (27%) severe compromised testes were seen from day 45 to day 540 postoperatively. Of the control group, 36 testes exhibited normal appearance ($P < 0.001$). Of the SVCD testes with mild or severe spermatogenic arrest in group II and III, there was only mild reduction in testicular size ($P > 0.05$), but a strikingly reduced STA ($P < 0.01$). The Johnsen

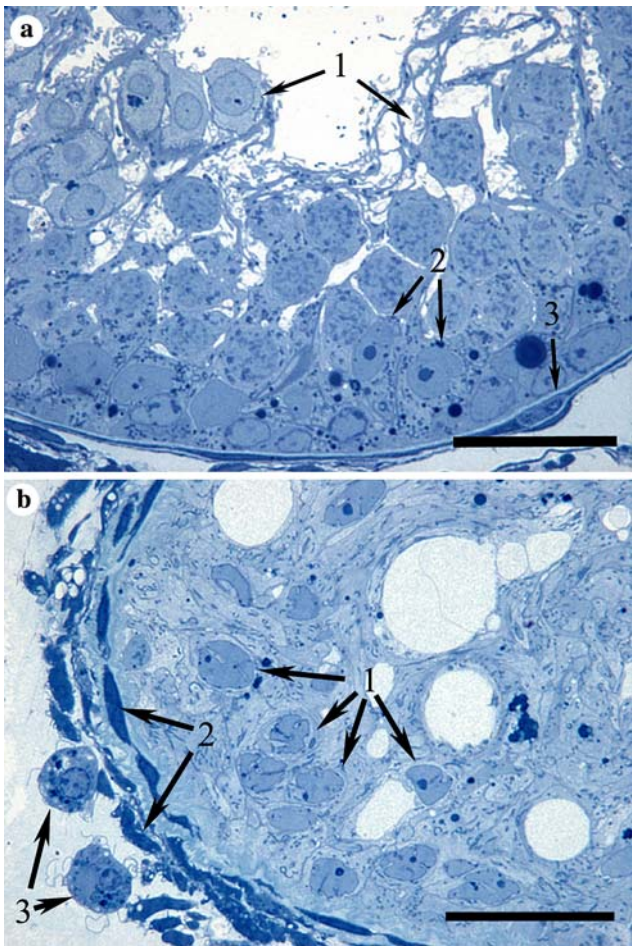

Fig. 2 **a** Severe spermatogenesis arrest. *1* Spermatocyte and spermatide lost tight junction with other germ cells. *2* Sertoli cells left basal lamina. *3* Myoid cell hyperplasia. Bar = 30 μm. **b** Sertoli cell only tubule. *1* Sertoli cells engulfing apoptotic germ cells. *2* Evidently thickened basal lamina composed of hyperplastic myoid cells. *3* Increased and active macrophages. Bar = 30 μm

score was significantly reduced on day 9–45 ($P < 0.001$), and on day 180 ($P < 0.01$).

In the case of SVCD testes that presented normal, mild or severe spermatogenic arrest, normal histology was observed in the contralateral testes in group II. However, in the case of SVCD testes with atrophy, evidently histological changes were seen in the contralateral testes. In a total of 15 investigated contralateral testes, mild spermatogenic arrest occurred in 13 cases from day 45, postoperatively. Severe spermatogenic arrest was established in two on day 360 and 540, postoperatively.

Parallel to the spermatogenic arrest that occurred in the involved testes, Leydig cell hyperplasia was a common sign. With time, these hyperplasia reached the size of adenomas ($n = 42$; Fig. 3). However, only two LC hyperplasia were found among the age-matched controls ($P < 0.001$). Without contralateral testes (group III), the obvious differences from those seen in group II were that Leydig cell hyperplastic lesions were exclusively confined to the all 23 involved testes from day 45 postoperatively.

Discussion

In the clinic, laparoscopy has given better surgical outcome with less morbidity than open surgery in the management of impalpable testis and varicocele [3–5]. In our initial experiment, it was found that the testes of prepubertal rats (aged 20–30 days) were susceptible to injury. Compared to open surgery (longer duration, larger incision and abdominal cavity exposure to air), laparoscopic surgery was less likely to affect the well-organised seminiferous epithelium (tight junction between the germ cells and Sertoli cells), and it was easier for the rats to recover from the operation (data were not presented). Thus, laparoscopic SVCD in 30-day-old rats might mirror the clinical situation of laparoscopic SVCD.

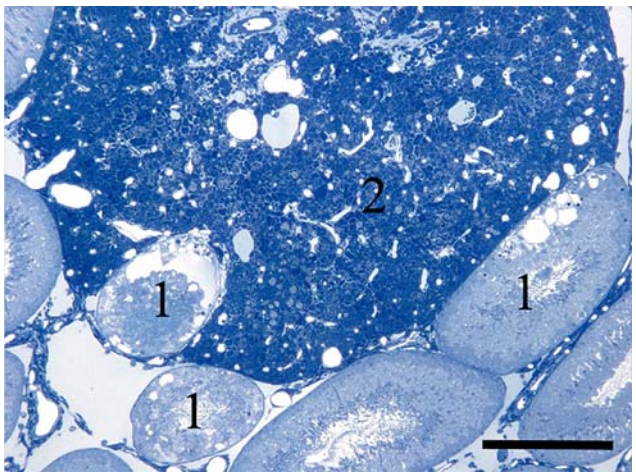

Fig. 3 Leydig cell adenoma in mild atrophic testis. *1* Mild atrophic tubules. *2* Leydig cells adenoma. Bar = 300 μm

There were varying incidences of testicular atrophy following Fowler-Stephens orchidopexy in different reports, and the criteria for evaluating testicular atrophy were not clearly described [4, 16]. If only evident volume reduction of the operated testis was defined as atrophy, the ratio of complete spermatogenic arrest in the present study could be comparable to the atrophic ratio that is widely accepted (51 vs 20–50%) [4, 16] after Fowler-Stephens orchidopexy. Is that mean the testicular atrophy must be the testis loss all the spermatogenesis? Recent reports of laparoscopic Palomo procedure revealed favourable outcomes [17, 18]. However, can it be considered as normal if the testis reveals normal or mild decreased volume after SVCD procedure? It has been observed that 40% of the testes were atrophied and 48% were volume decreased in addition after two-staged orchidopexy [19], in which the authors did not confirm whether the decreased volume of testes was considered to be atrophy. In the present study, apart from the atrophic testes with evident size reduction, only 15% SVCD testes showed normal histology; 34% SVCD testes revealed normal or mild size reduction, but evidently decreased ST area and Johnsen score with partial (24%) or severe (10%) impaired spermatogenesis from peripuberty to middle age. These testicular conditions may correlate to low sperm counts. This result agrees with an experiment subsequent to SVCD in young rats [20]. Thus, it could be presumed that after SVCD many compromised spermatogenesis, which will persist to peripuberty or even middle age, might be ignored in the clinic, if the testicular atrophy is evaluated by testicular volume alone.

On observation of paraffin sections within a relatively short period (18–60 days), some authors noted that ligation of spermatic vessels could produce varying degrees of injury of the operated testicle, but did not affect contralateral spermatogenesis [21–23]. However, based on the perfusion fixation and observation of the semithin sections, the present study found that from day 45 postoperatively, impaired spermatogenesis of contrallateral testes occurred when the SVCD testes showed atrophy, which might result in fertility due to mild disturbed spermatogenesis in postpuberty (45–180 days postoperative) or might result in subfertility or infertility owing to significantly impaired spermatogenesis (Sertoli cell only tubules) in middle age (360–540 days postoperative). The mechanism of this phenomenon is unknown. It might be the same as that of unilateral testis torsion for autoimmune etiology, but not a reflexive sympathetic response, because it appeared from 45 days postoperatively. However, the mechanism of SVCD and testis torsion should be different, as the former does not interrupt the vas defence and its vessels. Some causes were postulated for the subfertile or infertile

cryptorchid population treated in childhood, such as congenital degeneration or congenital disorder of the hypothalamic–pituitary–testicular hormonal axis. However, the sub- or infertility could also be caused by SVCD, a fact that to our knowledge was not mentioned previously. On the other hand, the results of the present study implies that the atrophic testis after the SVCD should be removed as early as possible, in order to rescue the contralateral testis.

Progressive degeneration of germ cells and dysplasia seen in cryptorchid testes is thought to be related to an increased risk of malignancy [24, 25]. Most human testicular tumours are either of germ or Sertoli cell origin. Tumours of Leydig cell origin constitute only about 2–3% of human testicular tumours [26]. In the present study, Leydig cells hyperplasia and adenoma developed frequently in the mild, severe or complete spermatogenic arrest testes, indicating a close relation between spermatogenic arrest and Leydig cell hyperplasia or adenoma. In Wistar rats, the incidence of Leydig cell tumours was reported to be 4–7% [27, 28]. Whether the testicular tumours originate from germ cells or Sertoli cells in humans and from Leydig cells in rats, and whether they share a common aetiology in compromised spermatogenic arrest testes are unknown.

Conclusions

The laparoscopic dissection of spermatic vessels in prepubertal rats could disturb spermatogenesis in most cases with differing severity, although only complete spermatogenic arrest testes showed evident decrease in size. This impaired spermatogenesis could persist from peripuberty to middle age in operated testes, and even injured contralateral testis in the case of operated testes showed complete spermatogenesis arrest. This study showed that laparoscopic SVCD might be of high risk in compromising the involved testis.

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