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Evaluation of cold ischemia for preservation of testicular function during partial orchiectomy in the rat model

Erin R. McNamara^{a,*}, Ramiro J. Madden-Fuentes^a, Jonathan C. Routh^a, Douglas Rouse^b, John F. Madden^c, John S. Wiener^a, Harry G. Rushton Jr^d, and Sherry S. Ross^a

¹Division of Urology, Department of Surgery, Duke University Medical Center, Durham, NC, USA

²Division of Animal Laboratory Resources, Duke University Medical Center, Durham, NC, USA

³Department of Pathology, Duke University Medical Center, Durham, NC, USA

⁴Department of Urology, Children's National Medical Center, Washington, DC, USA

Abstract

Objective—We hypothesized that cold ischemia during partial orchiectomy would lead to higher serum testosterone levels and preservation of testicular architecture than warm ischemia in a prepubescent rat model.

Materials and methods—Eighteen prepubescent male Sprague–Dawley rats were randomized to three different surgical groups: sham surgery, bilateral partial orchiectomy with 30 min of cord compression with cold ischemia, or bilateral partial orchiectomy with 30 min of cord compression with warm ischemia. Animals were killed at puberty, and serum, sperm, and testicles were collected. Histological tissue injury was graded by standardized methodology.

Results—Mean serum testosterone levels were 1445 ± 590 pg/mL for the sham group, 449 ± 268 pg/mL for the cold ischemia group and 879 ± 631 pg/mL for the warm ischemia group ($p = 0.12$). Mean sperm counts were 2.1×10^7 for sham, 4.4×10^6 for cold ischemia, and 9.9×10^6 for the warm ischemia groups ($p = 0.48$). Histological evaluation revealed significant difference in tissue injury grading with more injury in the cold ischemia than in the warm ischemia group ($p = 0.01$).

Conclusions—In our preclinical rat model, we found no benefit for cold ischemia over warm ischemia at 30 min.

Keywords

Testes; Ischemia; Orchiectomy; Testicular neoplasm; Rats

Conflict of interest

None of the authors have any conflict of interest, financial or otherwise, to disclose that would bias this research.

Introduction

There is growing enthusiasm for testis-sparing surgery (TSS) in both pediatric and adult urology patients. Several studies have demonstrated that the majority of prepubescent tumors are benign, thus arguing for a more conservative approach than radical orchiectomy [1,2]. This coupled with the desire to improve long-term function has led to adoption of a TSS approach in benign appearing prepubescent tumors to better preserve testicular function, spermatogenesis, and normal pubertal development [3,4]. In the post-pubertal patient, reported indications for TSS include a solitary testicle, bilateral synchronous malignancy, desire for paternity and independence from androgen supplementation [3–5].

The technique for TSS was first attributed to Richie in 1984 and further described by Weissbach in 1995 [6]. The testicle is delivered through an inguinal approach, the spermatic cord is clamped and the testicle is placed in ice slush to induce cold ischemia. The tunica is opened; the mass is excised and sent for frozen pathological evaluation. After pathological examination confirms that the margins are negative, the tunica is then closed.

In the pediatric literature, there are reports of cord occlusion with or without cold ischemia [9,10]. Historically cold ischemia has been used in both renal and testicular surgery in an attempt to preserve organ function. In the renal literature, studies differ on the role of warm versus cold ischemia and factors such as time of ischemia seem to play a more important role in preserving function [11]. In TSS, it is unclear if cold ischemia is beneficial.

Preclinical adult models evaluating the role of cold ischemia during testicular torsion have shown that cold ischemia is protective over 4 h of torsion [7,8]. One study looked at hypothermia and ischemia in a prepubertal rat testicle and found that histology in the cold ischemia group was preserved compared to warm ischemia as early as 30 min [9]. We, therefore, hypothesized that cold ischemia during prepubescent partial orchiectomy would improve post-pubertal serum testosterone levels, sperm count, and histological architecture when compared to warm ischemia in a rat model.

Methods

Animals and partial orchiectomy

Eighteen male prepubescent Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA), obtained after weaning at day of life 21, were utilized according to experimental protocols approved by our Institutional Animal Care and Use Committee. The animals were randomized to one of three groups. Rats in Group 1 underwent sham surgery ($N = 4$ rats, 8 testicles), Group 2 underwent bilateral partial orchiectomy with 30 min of cord compression and cold ischemia via ice slush ($N = 7$ rats, 14 testicles), and Group 3 underwent bilateral partial orchiectomy with 30 min of cord compression and warm ischemia (no ice slush, $N = 7$ rats, 14 testicles).

All surgeries were performed under loop magnification with animals maintained under 2% inhaled isoflurane anesthesia. The groin and scrotum were shaved and prepped in a sterile fashion. A low midline incision was made and the spermatic cords were identified bilaterally

to deliver the testicles into the field. A mini-vessel loop (Sterion) was placed around the cord and a micro-vascular clamp (bull-dog) was used to tighten the loop and occlude the spermatic cord. This step was designed to mimic Penrose drain occlusion of the spermatic cord in pediatric and adult testicular surgery. In group 2, each testicle was placed in ice slush for hypothermia. The ice slush was prepared in deep Petri dishes and placed next to the body on either side so the testicles were completely immersed. At 15 min of cold ischemia, the tunica was incised with a 15-blade and a portion of the tubules excised and placed in formalin. The tunica was then closed with 6-0 and 7-0 absorbable suture. These same steps were performed with the contralateral testicle in the cold ischemic group. The warm ischemic group underwent the same procedure without ice slush. After 30 min of ischemia time, the vessel loop was removed. The testicles and spermatic cord were replaced in their correct anatomic location without any evidence of ischemia or congestion. The midline incision was closed, and the rats were awakened from anesthesia.

Five weeks after the initial surgery, when the animals were post-pubertal, the animals were euthanized by carbon dioxide inhalation and bilateral thoracotomy. Blood was collected immediately by cardiac puncture. The serum was sent for testosterone levels (Texas A&M Veterinary Medical Diagnostic Laboratory, College Station, TX, USA). Both testicles were harvested through a midline incision and were placed in 10% formalin. The epididymitides were harvested and processed for sperm counts, in a standard fashion, by a veterinarian, blinded to the groups [10].

Histopathology

Testicles were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (5–7 μm thick), and stained with hematoxylin and eosin. The histologic parameters studied are listed in Table 1, as described by Cosentino and colleagues [11–13]. Each testis was scored from 0 to 4 for each parameter, with the higher numbers indicating a more pathologic condition. Testicular tissue injury was determined in a blinded fashion by a uropathologist (J.M.) and standardized grading can be seen in Table 2. Representative slides of the testicular histology are seen in Fig. 1. Each histologic parameter was compared between the cold ischemia and warm ischemia groups and total mean histology score was compared between the two groups [13].

All images were taken at the Duke University Light Microscopy Core Facility using the Zeiss Axio Imager system with Metamorph software (Molecular Devices, Inc., Sunnyvale, CA, USA).

Statistical analysis

Mean and standard deviation (SD) were calculated for all continuous variables. Data were analyzed for differences among groups using ANOVA, the *t* test or the Fisher exact test based on data characteristics (Graph Pad Prism 6).

Results

All rats survived surgery. Serum, sperm, and testicles were collected from all animals at time of euthanasia and weights were obtained. Group 1, sham rats, had a mean weight of

355.9 ± 50 g, Group 2, cold ischemia, had a mean weight of 339.6 ± 65.4 g, and Group 3, warm ischemia, had a mean weight of 345.1 ± 58.1 g. There was no difference between the three groups ($p = 0.9$).

Testosterone

Serum testosterone levels were compared among the three groups. Mean serum testosterone level in Group 1 was 1445 ± 590 pg/mL, in Group 2 was 449 ± 268 pg/mL, and in Group 3 was 879 ± 631 pg/mL. Between the three groups, there was a significant difference in serum testosterone ($p = 0.02$); however, serum testosterone levels were not significantly different between the cold and warm ischemia groups, which is what our hypothesis was testing ($p = 0.12$).

Sperm count

Mean sperm count in Group 1 was 2.1×10^7 sperm/mL, in Group 2 was 4.4×10^6 sperm/mL, and in Group 3 was 9.9×10^6 sperm/mL. Sperm counts were not significantly different between the cold and warm ischemia groups ($p = 0.48$).

Histology

The architecture of the cold ischemia testicles ($n = 14$) was compared to the warm ischemia testicles ($n = 14$) and compared to shams using Cosentino histology grades as described above. The sham testicles had a total mean histology score of 4 ± 5.9 . The total mean histology score among the cold ischemia testicles was 19.2 ± 7.6 . This was significantly different from the warm ischemia testicle mean score of 12.2 ± 5.9 ($p = 0.01$). All 12 histologic parameter scores are seen in Fig. 2. The four most disparate parameters between the two groups were (a) loss of spermatozoa and spermatids ($p = 0.01$), (b) disarray of germ cells ($p = 0.02$), (c) thickness of myoid cell layer ($p = 0.01$), and (d) fibrosis ($p = 0.06$). These histological changes were significantly more prevalent in the cold than in warm ischemia testicles.

Discussion

In our preclinical model, cold ischemia appeared to have no benefit over warm ischemia at 30 min. We found no significant differences in post-pubertal serum testosterone and sperm counts between the cold ischemia and warm ischemia groups after bilateral prepubescent partial orchiectomy. The total mean histologic grades were significantly worse in the cold ischemia group. There was notably more tissue damage in the cold ischemia testicles, specifically with regards to loss of spermatozoa and spermatids, disarray of germ cells, thickness of myoid cell layer, and fibrosis.

The majority of studies evaluating hypothermia and the ischemic rat testicle have focused on ischemia secondary to torsion over periods of ischemia ranging from 2 to 8 h. Miller et al. [8] reported that at 2 h of ischemia, there was no difference in the average number of germ cell layers between cold and warm ischemia; however, at 4 h, there was a significant difference, favoring cold ischemia. Haj et al. [7] studied external scrotal cooling during 4 or 8 h of spermatic cord torsion and found that cooling decreased both immediate and late

reperfusion damage. Almodhen et al. [9] is credited with the first study evaluating the prepubertal rat testes and reported that as early as 30 min after torsion there were differences after puberty in testicular length, seminiferous tubular diameter, and germinal cell layers between the cold and warm ischemia groups, with increased preservation in the cold ischemic group.

Our study differs from these reports in three important factors. First, this study was specifically designed to mimic the disruption of the tunica and tubules in a partial orchiectomy. Second, we employed a histological grading system described in the literature to evaluate 12 important testicular parameters. The 12 components of these scores can be interpreted separately and/or in combination to understand detailed changes to testicular structure and the overall effect of ischemia on the testicle. Lastly, while histology is extremely important to understand the ischemic effects on structure and downstream function, the ultimate parameters of testosterone and sperm production are critical clinical outcomes related to fertility and pubertal development.

Within the renal literature, there is controversy about cold and warm ischemia during partial nephrectomy, and our results suggest that the same may hold true for partial orchiectomy. While other studies have looked at ischemia in relation to torsion, this is the first preclinical model studying partial orchiectomy using cold ischemia with cord occlusion. Practice patterns among urologists vary in terms of their use of cold ischemia during TSS, and, thus, preclinical evidence may help guide clinical practice.

Our data must be interpreted in the context of the study design. This is a pilot study and, therefore, is limited by the small number of animals and statistical power. In order to see a difference between serum testosterone and sperm count, we would need more samples. There seems to be a clear trend that overall the serum testosterone and sperm count are lower in the cold ischemia group compared to the warm ischemia group, but we cannot make a definitive conclusion with the small numbers. The significant difference in histology scores does highlight the differences between cold and warm ischemia and suggests that cold ischemia results in more damage to the testicle. In addition to the issue of sample size, a recent study questioned the use of rat testicles for andrology and associated research, noting that among 600 “normal rat testicles” approximately 2.5% had testicular abnormalities included interruption of spermatogenesis, partial germ cell arrest, progressive hypospermatogenesis, seminiferous epithelia vacuolation and inflammatory status [14]. However, to date, no robust alternative exists to evaluate histological outcomes in concordance with serum and sperm parameters. We did not examine paternity outcomes in the study rats, so we cannot determine whether the differences noted truly impacted eventual fertility. Also, we did not study the temperature of cold ischemia, and, perhaps, cooling of varying degree may be more deleterious.

Our data does not support our hypothesis that cold ischemia is protective to the testicle undergoing TSS. In fact, cold ischemia in this setting may result in worse long-term histological parameters. While not statistically significant, serum testosterone and sperm counts were lower in the cold ischemia group suggesting that the overall outcome from cold ischemia during TSS may be detrimental to all aspect of testicular architecture and function.

Further research should be done to study this in larger samples to confirm or refute our findings, as this is important for surgical planning. While application of this study to the clinical realm would be preliminary at this point, this study does highlight the need for collection of long-term data on patients undergoing TSS with cold or warm ischemia, especially in the rare but reported bilateral cases. Such information is paramount to clearly define optimal techniques to achieve the best outcomes.

Conclusion

The endpoints of testosterone production, sperm count, and histology of the testes are relevant within the pediatric population undergoing TSS. Using these parameters, we found that cold ischemia was not protective of testicular histology and function, but we did demonstrate evidence of long-term testicular function after prepubescent partial orchiectomy. Fertility is also an important outcome that should be assessed as we continue to pursue TSS in the pediatric population.

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Specimen A- Grade 0 injury Normal seminiferous tubule morphology (H&E,10x)

Specimen B- Grade 3 injury Disordered, sloughed germinal cells with less distinct borders (H&E, 10x)

Figure 1.
Testicular histology.

Figure 2.
Mean histology scores.

Table 1

Histologic parameters examined.

<i>Seminiferous tubules</i>
Loss of spermatozoa and spermatids
Absence of germ cell layers
Degeneration of germ cell layers
Disarray of germ cell layers
Rupture of tubules
Leydig cell reactions at ruptured tubules
Thickness of myoid cell layer
<i>Interstitial</i>
Leydig cell proliferation
Edema
Hemorrhage
Granuloma
Fibrosis

Table 2

Histology grading scores.

Grade	Description
0	Normal testicular architecture
1	Minimal change in germinal cell layer
2	Less orderly, non-cohesive germinal cell layers; closely packed seminiferous tubules
3	Disordered, sloughed germinal cells with shrunken, pyknotic nuclei; less distinct seminiferous tubule borders
4	Seminiferous tubules packed closely with coagulative necrosis of germinal cells