Modulation of Thrombosis Significantly Reduces Testicular Damage after Testicular Torsion in Rats: Anti-Thrombotic Treatment and Testicular Torsion

Michael Boettcher, Tobias A. Fuchs, Hansjörg Schäfer, Birgit Appl, Magdalena Trochimiuk, Miguel Jiménez-Alcázar, Bastian Tiemann, Roman Jung, Robert Bergholz, Konrad Reinshagen, and Georg Eschenburg

OBJECTIVE To evaluate the effects of thrombolysis and/or anticoagulation on testicular viability after testicular tortion (TT) was the aim of this study. It has been suggested that alterations of circulation during TT result in thrombus formation that might prevent sufficient perfusion after detorsion. Due to the narrow safety margin of testicular perfusion, even moderate disturbances in blood supply can cause major testicular damage.

METHODS In 112 rats, the right testicle was torsed for 3 or 6 hours. After detorsion and randomization, they received either enoxaparin, alteplase, both, or placebo, according to their subgroup. Thrombus formation was accessed via D-dimers, pDNA, oxidative testicular damage was evaluated via glutathione peroxidase and malondialdehyde, and cellular damage via inhibin B, testosterone, histological analysis (Johnsen score, Cosetino grading), and TUNEL assay.

RESULTS One hundred and twelve rats were included in the study. The treatment with alteplase or enoxaparin showed significantly less testicular damage and significantly improved Sertoli cell function. Enoxaparin significantly reduced oxidative impairment.

CONCLUSION The results of the study indicate that TT induces thrombus formation and demonstrate that modulation of thrombosis significantly ameliorates testicular damage in rats. Hence, this treatment option after TT ought to be evaluated in humans. UROLOGY 88: 227.e1–227.e7, 2016. © 2016 Elsevier Inc.

Testicular torsion (TT) is a common urological emergency involving rotation of the testis and twisting of the spermatic cord, which restricts blood flow to the affected testis.[1,2] The testis receives its blood supply through a long artery leading to high vascular resistance.[3] Thus, even moderate disturbances in organ circulation can cause testicular malfunction.[4] Prolonged testicular ischemia triggers oxidative stress and cellular damage, which get aggravated after blood flow is reestablished via reactive oxygen species and systemic inflammatory response.[5] As a result, TT seriously interferes with subsequent **spermatogenesis** in about half the patients and sperm counts <20 million/mL are found in one third of them after TT.[6] Consequently, focus of most studies has been to ameliorate TT treatment by reducing the ischemia-reperfusion-injury, that is with antioxidative substances or improving microvascular blood flow with rheological substances.[5]

One possible aspect of testicular impairment has been neglected. It has been shown that alterations of the circulation during TT result in thrombus formation.[7,8] Compression of the pampiniform plexus during TT leads to venous congestion and edema that in later stages impairs the microarterial system because the tunica albuginea is an inelastic shell, which limits compensatory expansion of the testis, resulting in ischemia.[9] Venous congestion leads to clogging of the microvessels via activation of the intrinsic coagulation pathway. Furthermore, hypoxia upregulates both intrinsic and extrinsic coagulation pathways and contributes to thrombus formation.[10] A recent study reported elevation of D-dimer levels 4 hours after TT in

Financial Disclosure: *The study was supported by a research grant (BBSTD-14-00016) of B.Braun foundation (Melsungen, Germany).*

From the Department of Pediatric Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; the Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; the Department of Pathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; and the Department of Experimental Animal Research, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Address correspondence to: Michael Boettcher, M.D., Department of Pediatric Surgery, UKE Medical School, Martinistrasse 52, 20246 Hamburg, Germany. E-mail: m.boettcher@uke.de

Submitted: August 25, 2015, accepted (with revisions): November 3, 2015

rats, suggesting activation of intravascular coagulation in TT.[11] Ultimately, thrombus formation during TT might contribute to testicular damage due to a no-reflow phenomenon with reduced microcirculation after restored macrocirculation. Hence, the aim of this present study was to evaluate the effects of modulation of thrombosis on testicular viability after TT.

METHODS

Study Design

The study was approved by the Hamburg State Administration for animal research (146/13). A total of 112 male adolescent Wistar rats (Charles River, Wilmington, USA) 6-7 weeks old were utilized for this experimental model. All environmental parameters within the animal facility complied with the German guide for the care and use of laboratory animals (Tierschutzgesetz).

Animal Procedures. For better standardization, one operator performed all operations. Anesthesia was induced with 5% isoflurane and maintained with 2-3% isoflurane delivered through a facemask. In all rats, 5 mg/kg carprofen and 0.02 mg/kg buprenorphine for analgesia and 10 mg/kg enrofloxacin as prophylactic antibiotic therapy were administered subcutaneously. At the beginning of the experiment and after 1-5 hours of torsion, 0.2 mL blood was taken after retro-orbital sinus sampling.

The rats were placed in a dorsal decubitus position. Preoperative antisepsis was performed with phenoxyethanol. After a small vertical incision in the middle of the scrotum, the right testis was exposed, twisted around 360° or 720° (depending on the group) and fixed in this position on two points with a 6 × 0 Prolene Suture (Ethicon, Norderstedt, Germany). The scrotum was closed using 5 × 0 Prolene. After 3 or 6 hours (depending on the group), the scrotum was reopened and the right testis was repositioned in its original position. The scrotum was closed using 5 × 0 Prolene.

The rats were divided randomly into four treatment groups with two different durations of torsion (3 or 6 hours) and two different degrees of torsion (360° or 720°):

I. Treatment group controls: a single bolus of 0.5 mL NaCl 0.9% subcutaneously.
II. Treatment group enoxaparin: a single bolus of 200 IU/kg bodyweight of enoxaparin sodium (Clexane, Sanofi-Aventis, Germany) subcutaneously.
III. Treatment group alteplase: a single bolus of 0.9 mg/kg t-PA (Alteplase, Boehringer Ingelheim, Germany) via tail vein injection.
IV. Treatment group enoxaparin and alteplase: dosage and route as above.

Dosages of enoxaparin and alteplase were deduced from thrombolysis protocols using the dosage translation technique of Haelewyn et al[12] and Reagan-Shaw et al.[13] The medication was administered after testicular repositioning. After discontinuation of anesthesia, the animals were housed in the animal facility. For pain control, all rats were fed a daily dose of 0.1 mg/kg bodyweight meloxicam within baby puree. On day 7, the rats was anesthetized with 5% isoflurane using the same setup as described above. In all rats, bilateral orchiectomy was performed and 2 mL blood was taken after intracardial puncture. Finally, they were euthanatized with intracardial injection of 40 mg/kg body weight thiopental.

Tissue Preparation and Evaluation. The excised testicles were weighed and a small slice of the native testes was immediately frozen at −80 °C. Another slice was placed in RNAlater solution (Life Technologies, Darmstadt, Germany), stored for 24 hours at 4°C and was then frozen at −80°C. The remaining tissue was fixed in Bouin's solution for its excellent preservation of nuclei and chromosomes during meiosis.[14] The specimens were embedded in paraffin, cut in 5 μm thick sections, stained using hematoxylin and eosin and periodic acid–Schiff. A blinded pathologist examined the testis using light microscopy. At 5 separate and randomly chosen places in the same histologic section, the following aspects were accessed:

I. The mean tubulus diameter accessed by measuring from the basement membrane to the lumen of the tubule at 90°C, 180°C, 270°C, and 360°C.
II. Histopathological damage to the seminiferous tubules was quantified as described by Cosentino et al.[15]
III. Maturity of germinal epithelium was graded by assignment of the modified Johnsen testicular biopsy score.[16]

TUNEL Assay. A TUNEL assay to detect deoxyribonucleic acid (DNA) fragmentation in cell nuclei (a marker of apoptosis in testicular tissue) was performed by using In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol.[17] Stained nuclei in 20 circular seminiferous tubule cross-sections per testis tissue section were assessed in a standardized and automatic fashion using the Nucleus counter plugin by ImageJ 2.0.0 (Fiji distribution) and averaged for each testis. The results are expressed as apoptotic cells per tubule cross-section.

Malondialdehyde (MDA) Assay. Tissue MDA activity (a marker of lipid peroxidation) was assessed using the Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich, MO, USA) according to manufacturer's instructions. The results are expressed as nmol/g tissue.

Glutathione Peroxidase (GPx) Assay. Tissue GPx activity (a marker of systemic antioxidant status) was measured using Glutathione Peroxidase Assay Kit (Cayman, MI, USA) according to manufacturer's instructions. The results are expressed as U/mg protein.

Blood Analysis. Blood samples were taken immediately before the intervention and 7 days after the operation. All