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Original article

Does penile tourniquet application alter bacterial adhesion to rat urethral cells: an *in vitro* study

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ABSTRACT

Purpose: To investigate the effects of penile tourniquet (PT) application on bacterial adhesion to urothelium.
Methods: Fifty-six rats were allocated into control group (CG), sham group (SG), PT group (PTG). No intervention was applied in CG. A 5 mm-length urethral repair was performed in SG and PTG. In PTG, a 10-min duration of PT was applied during the procedure and the tissue oxygenation monitor was used to adjust the same degree of ischemia in all subjects. Samples were examined for wound healing parameters and tissue levels of inflammatory markers, eNOS, e-selectin, and ICAM-1antibodies. The adhesion of *Escherichia coli* to urothelium was investigated with *in vitro* adhesion assay.
Results: Inflammation was higher and wound healing was worse in SG than CG and in PTG in comparison to CG and SG ($p < 0.05$). The endothelial damage, as shown by eNOS expression, was significantly higher in PTG compared to CG and SG ($p < 0.05$). The staining with ICAM-1 and e-selectin antibodies, showing increased inflammatory response to bacterial adhesion, was significantly higher in PTG compared to CG and SG ($p < 0.05$). *In vitro* urethral cell proliferation was achieved only in CG and SG revealing significantly increased adhesion in SG compared to CG ($p < 0.05$). The PT application caused endothelial corruption and prevented cell proliferation in cell culture.
Conclusion: The PT application does not improve wound healing and increases bacterial adhesion molecules in penile tissue. The *in vitro* assays showed that PT causes severe endothelial damage and inhibits endothelial cell proliferation.

A tourniquet is a compressing device used to control the blood flow distal to it. The application of tourniquet for maintaining bloodless surgical field dates back centuries ago when used during amputations [1]. The application of tourniquet application has also been used in penile surgery since Redman introduced the usage of rubber band as penile tourniquet (PT) in hypospadias surgery [2]. In addition to rubber band, penrose drain, which was favored by Crawford et al. [3], soft latex catheter, and rolled margin of a latex glove have also been used as PT in penile surgery [1,4].

Although PT is very useful in maintaining bloodless surgical field and obtaining better results in penile surgery, it also has several adverse effects. These adverse effects are classified as systemic or local effects. The systemic effects of PT can be negligible since they cause minor changes in blood volume. Therefore, the local effects are more prominent and important in penile surgery. The local effects are due to both mechanical compression to the tissues beneath and distal to tourniquet and ischemia of the tissues distal to tourniquet [1,5]. Additionally, a reperfusion injury may be observed after the removal of tourniquet. The incidence of the actually observed complications of PT is unknown. Penile edema, impaired wound healing and graft failures after hypospadias surgery were reported as complications of PT [6,7]. The complications that might lead to infection following the application of PT in hypospadias surgery have not yet been studied.

Many researchers investigated the adverse effects of PT including effects on urothelial changes [4], nitrergic responses [8], histopathological alterations and wound healing [9], inflammation and dysregulation of the expression of growth factors [10]. However, the effect of PT on bacterial adhesion to urothelium has not been investigated. Therefore, we

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conducted an experimental and *in vitro* study to investigate the effects of PT application on bacterial adhesion to urethral endothelium besides the effects on biochemical and histopathological alterations.

1. Materials and methods

All experiments were performed following the approval by the Local Ethical Committee (2014/04–14/48) and were performed according to the recommendations of the committee for laboratory animal care. Fifty-six Wistar albino male rats, weighing 300 ± 10 g, were included in the study. All rats were kept in standard cages at 22 °C room temperature and on a 12 h day/night cycle and were fed *ad libitum*.

The animals were allocated into the control group (CG, n = 8), the sham group (SG, n = 24), and the PT group (PTG, n = 24). SG and PTG were allocated into 3 subgroups according to the time of tissue harvesting; 1 h (SG-1, PTG-1), 24 h (SG-2, PTG-2), and 72 h (SG-3, PTG-3) after urethral repair.

The animals were anesthetized with intraperitoneal ketamine hydrochloride (50 mg/kg, Ketalar, Eczacıbası, Istanbul, Turkey) and xylazine hyrochloride (5 mg/kg, Alfazyne, Alfasan Int BV, Woerden, Holland). After anesthetization rats were placed at supine position. Their genitalia were prepared with poviodine-iodine. In CG, penectomy was performed without any intervention. In SG and PTG, the penile skin was incised circumferentially at the level of mucosa and hairy skin junction on penis and degloved in order to reach the urethra. The urethra was catheterized with a 23 Gauge angiocath. A 5 mm length vertical incision was performed on the ventral face of urethra, and then repaired with two interrupted 5/0 polyglactine suture (Vicryl®). In PTG, a rubber circular band was applied to the degloved penile base for 10 min during the operations. After 10 min, PT was removed. The procedures lasted 10 min in each animal, so there was no need for the application of a second PT. In PTG, the tissue oxygenation monitor (MoorVMS-OXY, Moor Instruments Ltd., Millwey Axminster Devon, UK) was used to adjust the same degree of ischemia in all subjects. The tissue oxygenation was measured before PT, during PT, and after the removal of PT and were expressed as percentages.

Samples were examined biochemically (the expression of nitric-oxide-NO, malondialdehyde-MDA, myeloperoxidase-MPO, tumor necrosis factor-alpha-TNF-α, interleukin 1 beta-IL-1β), histopathologically (wound healing scores) and immunohistochemically by staining with antibodies against e-selectin, ICAM-1 and eNOS. Urethra were isolated in each group and cultivated for *in vitro* assays to investigate bacterial adhesion to urothelium. All animals were sacrificed by exsanguination at the end of experiments.

1.1. Biochemical examination

The tissue levels of NO, MDA, MPO, TNF-α and IL-1β of the samples were examined. All samples were kept at −80 °C. Then, samples were washed with 0.9% NaCl at 4 °C and homogenized (Labor Technique, Müllheim, Germany). Homogenized tissue samples were centrifuged at 1.500×*g* for 10 min at 4 °C. Supernatants were used for the determination of MDA, total NO, MPO and protein levels by the method of Lowry et al. [11].

NO levels were measured by a spectrophotometric method as described by Miranda et al. [12] and the results were expressed as μM/g protein. MDA levels, indicating lipid peroxidation, were measured by the method described by Armstrong and Al-Awadi, which was modified from the Yagi method and the results were expressed as nM/mg protein [13]. MPO levels were determined using a commercially available quantitative enzyme linked immunoabsorbent assay (ELISA) kit (MPO-rat ELISA KIT/SEA601RA, USCN Life Science Inc., Houston, USA) and the results were expressed as ng/mg protein. TNF-α levels were determined by using the ELISA kit (TNFa-rat ELISA KIT /SEA133RA, USCN Life Science Inc., Houston, USA) and the results were expressed as ng/mg protein. IL-1β levels were also determined by using a quantitative ELISA kit Interleukin 1 beta (IL1B-rat ELISA KIT/SEA563RA, USCN Life Science Inc., Houston, USA) and the results were expressed as pg/mg protein.

1.2. Histopathological examination

The samples were fixed with 10% formalin and embedded in paraffin blocks vertically. Tissues were sectioned in 4–5 μm pieces and were stained using the routine hematoxyline-eosine and Masson-trichrome staining. The specimens were examined under a light microscope (Lecia DM 2500, Germany), which has the feature of Leica Microsoft Systems Framework vision system (LAS V 4.0) by the same pathologist who was blind to the study.

The samples were evaluated for inflammation by the examination of the quantitative density of inflammatory cells. The neovascularization was investigated by the density of the newly formed branched capillary vessels with prominent endothelium, and fibrosis was investigated by the examination of the accumulation of collagen and the proliferation of fibroblasts. The accumulation of polymorphonuclear cells and mononuclear cells reflecting inflammation was assessed as follows: not present or normal in number (0 points), slight increase (1 point), marked infiltration (2 points), or massive infiltration with the formation of micro abscesses (3 points). The neovascularization was scored as; 0: no vascular proliferation, 1: mild vascular proliferation, 2: moderate vascular proliferation, and 3: intense vascular proliferation. In the assessment of fibrosis, the quantitative increase of young fibroblasts and the presence of collagen were evaluated together. Samples were evaluated and scored as 0 if fibrosis was absent, as 1 for slight, as 2 for moderate, and as 3 for dense fibrosis and hyalinization. Therefore, the higher total pathology score indicated worse wound healing.

1.3. Immunohistochemical examination

Immunohistochemical studies were performed automatically in the Bond Max Equipment (Leica Microsystems Inc., Wetzlar, Germany). The antigen retrieval steps were performed in Bond-Epitope Retrieval Solution 1 (AR9961) for e-selectin antibody (Abcam/ ab185698, 1:100), ICAM-1 antibody (Abcam/ ab124759, 1:100), and eNOS antibody (Abcam/ab5589, 1:100) at 100 °C. The detection was carried out with Bond Polymer Refine Detection kit (LOT 22711). The stained slides were dehydrated and covered with mounting medium (DAKO/s3023, Denmark) and cover-slips. Non-immune mouse serum served as a negative control and Mayer's hematoxylin was used as the counterstain. The cytoplasmic staining was considered as positive staining for all antibodies. The positive staining was mainly observed in the endothelial cells, fibroblasts and inflammatory cells in the area of wound healing. The evaluation of immunohistochemical staining was graded arbitrarily as weak (+), moderate (++), and strong (+++).

1.4. Bacterial adhesion assay

The *in vitro* studies and the scanning electron microscopy (SEM) examinations were performed in Kırıkkale University, Laboratory of Research Center and Laboratory of Electron Microscopy. Urethra were isolated in each group under sterile conditions and cultivated for *in vitro* assays to investigate bacterial adhesion to urothelium.

Tissues were dissected into small pieces with sterile scalpels at the *in vitro* organ culture laboratory and were plated in 25 cm^2 flasks. Then, 89% DMEM (Dulbecco's Modified Eagles Medium), 10% FBS (Fetal Bovine Serum), and 1% penicillin streptomycin (to prevent contamination) containing medium were added into the flasks and incubated at 37 °C and 5% CO_2 for one week. Following cultivation to enable the proliferation of cells, the medium was removed and cells were mixed with 0.5 ml trypsin EDTA and incubated for 4 min. Cells were transferred into sterile tubes after inhibiting the trypsin activity and centrifuged at 2500 rpm for 2 min. The cell counts were performed after the pellets were reconstituted in 1 ml medium. Cells were seeded

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