Intraurethral Erythropoietin to Prevent Fibrosis and Improve Urethral Healing: An Experimental Study in a Rat Model

Muhammet Fatih Kilinc, Omer Gokhan Doluoglu, Pinar Eylem Eser, Yildiray Yildiz, Veli Mert Yazar, Ali Ayyildiz, and Sema Hucumenoglu

OBJECTIVE	To determine the effects of intraurethral erythropoietin (EPO) on an experimentally induced urethral injury in a rat model with respect to wound healing enhancement and the prevention of spongiofibrosis
MATERIAL AND METHODS	A urethral injury model was created by traumatizing the urethra of male rats with a tilted-tip insulin injector. Thirty rats were randomly separated into 3 groups of 10; Group 1 (control) received 0.9% saline solution twice a day, Group II received EPO 25 IU/kg once a day and 0.9% saline solution once a day, and Group III received EPO 25 IU/kg twice a day. All applications were made intraurethrally via a 24 ga catheter sheath. To investigate inflammation and spongiofibrosis and congestion of vessels in the lamina propria, the penises of the rats were harvested for histopathologic evaluation after a follow-up period of 14 days.
RESULTS	Histopathologic analysis revealed less fibrosis and inflammation and higher congestion of vessels in Group III that had received high-dose EPO. There was a significant decrease in both spongiofibrosis and inflammation and an increase in congestion in Groups II and III compared to the control group ($P = .001$, for all). In the comparison of Group II with Group III, no statistically significant differences were found in terms of these 3 parameters ($P = .5$, $P = .6$, $P = .27$, respectively).
CONCLUSION	The results of this study have shown that EPO has a preventive effect on spongiofibrosis and improve urethral wound healing in a rat model of urethral injury. UROLOGY 123: 297.e9−297.e14, 2018. © 2018 Elsevier Inc.

Urethral stricture occurs because of narrowing of the lumen in any segment of the urethra due to fibrosis developing as a result of trauma, infection or idiopathic reasons.[1] Although the etiology of many cases of urethral stricture remains unknown, the most common types of trauma are urologic interventions or instrumentation or perineal trauma. Minimally invasive treatments are currently often applied, but there are high rates of recurrence.[1,2] The reason for this is that whatever the etiology of the stricture, the accumulation of type 3 collagen which occurs more than is necessary in the injury site causes fibrosis.[3] To date, no medical treatment has been found that will prevent the formation of excessive fibrosis, increase the success rate of surgical treatments or decrease recurrence. Erythropoietin (EPO) is an essential hormone that stimulates erythropoiesis. This hormone stimulates angiogenesis and contributes to the maintenance of cell vitality in ischemic tissues.[4] EPO is also a tissue protective agent providing a significant contribution to the regulation of trauma, stroke, and inflammation.[5-8]

In consideration of the above-mentioned properties of EPO, it was thought that there could be an important effect on urethral healing. Therefore, the aim of this study was to examine the effect of EPO on urethral healing following trauma-induced injury in an experimental rat model.

MATERIAL AND METHODS

Approval for the study was granted by the Animal Ethics Committee. The study was conducted in the Animal Research Laboratory of Ankara Training and Research Hospital. A total of 30 male Wistar albino rats, each weighing 250-300 grams, were used in the study. All the animals were kept in separate cages at room temperature of 22°C and 50% humidity before the operation and in the postoperative period. On the study day, anesthesia of 50 mg/kg ketamine was administered to all the rats under

Figure 1. (A) Traumatization of the urethra using the tilted-tip insulin injector, (B) application of erythropoietin into the urethra with a 24 ga catheter sheath, (C) dissection of the penis. (Color version available online.)

Figure 2. (A) Areas of +++ severity fibrosis and collagenization observed in the control group with hematoxylin−eosin (HE) staining (x100 magnification), **indicates Masson Trichrome staining (x100 magnification). (B) >50 lymphocytes (Arrow) in an area at x 200 magnification (inflammation of +++ severity) on HE-stained slices observed in the control group. (C) High-dose EPO group urethra slices (x100 magnification) with HE staining, showing close to normal appearance not containing areas of fibrosis and collagenization, ** shows Masson Trichrome staining (x100 magnification). (D) Low-dose EPO group HE-stained slices (x100 magnification) showing increased vascular congestion (Arrow). (Color version available online.)
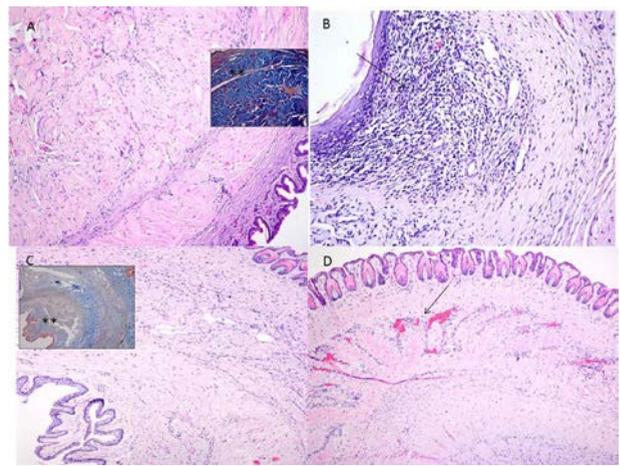
sterile conditions. The urethral trauma was applied via a curved-tip insulin injection needle (Fig. 1A). A 0.5-cm longitudinal cut was performed to the urethra at the 6 o'clock position. This cut was made starting from superficial tissue of the urethra to the deeper tissue that exceeds the muscles surrounding the urethra. This cutting maneuver was applied to all rats 4 times as standard with the curved-tip insulin injector needle.

The rats were then randomly separated into 3 groups of 10. The drugs at the predetermined doses were administered intraurethrally using a 24 ga catheter sheath (Fig. 1B). Group I was defined as the control group and the rats were administered 0.9% saline twice a day. The animals in Group II were administered 25 IU/kg EPO solution once a day and 0.9% saline twice a day. The animals in Group III were administered 25 IU/kg EPO solution twice a day. On day 15, the penis of each rat was degloved and penectomy was applied (Fig. 1C). All the specimens were placed in 10% formaldehyde and sent to the Pathology Department for histopathologic analysis. All the rats that survived throughout the study period were sacrificed at the end of the study (Fig. 2).

HISTOPATHOLOGIC ANALYSIS

Histopathologic analysis was performed under light microscope by a single independent pathologist blinded to the study groups. Until the day of macroscopic examination, the urethral tissues were fixed in 10% formol in a separate dish for each rat. During the macroscopic examination, the tissue samples were cut into squares at 3-mm intervals and embedded in paraffin blocks. Slices of 4 micron thickness were cut from the paraffin blocks and stained with hematoxylin and eosin (HE) and with Masson trichrome in the histochemical examination. The preparates were examined under light microscope at x100 and x200 magnification.

In the histopathologic examination of the tissues, evaluation was made of spongiofibrosis, inflammation, and congestion in vascular structures. In the histochemical examination, spongiofibrosis was examined with Masson Trichrome staining. Spongiofibrosis was evaluated as 0 = none; 1 + = ≤10% tissues with fibrosis; 2 + = 10%-49% tissues with fibrosis; 3 + = ≥50% tissues with fibrosis. Inflammation was evaluated as: 0 = none; 1+ = 5-10 lymphocytes/x200 magnification; 2+ = 11-50 lymphocytes/x200 magnification; 3 + = > 50 lymphocytes/x200 magnification. Congestion in vascular structures was calculated by counting the number of vessels with congestion in the tissue at each x100 magnification and dividing this by the number of total x100 magnification areas in the tissue: 0: none, 1 + = 1-3, 2 + > 3-6, 3 + = >6-10.

STATISTICAL ANALYSIS

Data analysis was performed using SPSS for Windows, version 11.5 software (SPSS Inc., Chicago, IL). Categorical variables were shown as the number of cases (n) and percentage (%). Categorical variables were analyzed with the Chi square test. A value of $P < .05$ was considered statistically significant.

RESULTS

All 30 rats survived throughout the study period and were included for evaluation. Spongiofibrosis, inflammation, and congestion of vessels in the lamina propria were evaluated in all the groups. Fibrosis of +++ severity was observed in 6 (60%) rats in Group I (control group), and in no rats of Group II (low-dose

Table 1. Comparison of the histopathologic analysis of the three groups

	Group I (Control)	Group II (Low-Dose EPO)	Group III (High-Dose EPO)	*P* Value
Spongiofibrosis (n, %)				.001
0	0	0	3(30%)	
+	0	7 (70%)	7 (70%)	
++	4 (40%)	3 (30%)	0	
+++	6 (60%)	0	0	
Inflammation (n, %)				.001
0	0	0	3 (30%)	
+	3 (30%)	10 (100%)	7 (70%)	
++	5 (50%)	0	0	
+++	2 (2%)	0	0	
Congestion (n, %)				.001
0	0	0	0	
+	8 (80%)	1 (10%)	0	
++	2 (20%)	7 (70%)	5 (50%)	
+++	0	2 (20%)	5 (50%)	

EPO) or Group III (high-dose EPO). No areas of fibrosis were observed in the normal urethra tissue of 3 rats in Group III (Table 1). A statistically significant difference was determined between the three groups in the three histopathologic conditions (*P* = .001). In comparison with the control group, both Group II and Group III were determined with statistically significant differences in respect of spongiofibrosis, inflammation, and congestion (*P* = .001, for all). In the paired comparisons between Group II and Group III, no statistically significant difference was determined in respect of spongiofibrosis, inflammation, and congestion (*P* = .5, *P* = .6, *P* = .27, respectively).

No side-effects were observed in any rat during the intraurethral applications.

COMMENT

Minimally invasive urethral surgery is an easy procedure in current clinical practice. Although the first recommended modality, it has a low long-term cure rate and higher recurrence rates compared to open urethroplasty.[9,10] Minimally invasive treatment has the disadvantages of an increase in the length and density of spongiofibrosis, which renders subsequent definitive surgical treatment more complex, leading to a risk of repeated anesthesia and repeated procedures increase the costs.[11]

The most important cause of low treatment success rates and the high risk of recurrence is known to be the increase in abnormal fibrosis.[3] The collagen concentration in fibrotic areas caused by urethral stricture has been reported to increase by 32%.[12] Several experimental and clinical studies have been conducted related to the application of drugs or substances with an antifibrotic effect to prevent this abnormal increase in fibrosis.[11,13-18] In the first studies, a steroid injection was applied to the stricture region.[13] Then, caprotil gel and halofuginone instillation were trialed for medical treatment of the urethral stricture.[14,15] In a clinical study by Mazdak et al.[16] the antifibrotic effect of Mitomycin-C was shown to be effective in preventing recurrence following internal urethrotomy. Sahinkanat et al.[11] conducted an experimental study and

reported that collagen increase was prevented with an injection of botulinum-A toxin as tensile distraction, which is another component of wound healing.

Another recent experimental study showed that the anti-inflammatory effect of dexpanthenol was effective in preventing urethral scar formation.[17] The gel combination of hyaluronic acid and carboxymethylcellulose applied with a Foley catheter between the urethra lumen to prevent tissue adhesion has been shown to reduce the recurrence rate.[18] However, none of these treatments to prevent urethral stricture has come into routine use.

During the process of dermal healing, the stages of inflammation, proliferation, maturation, and remodeling are observed. These stages occur in a longer process in the healing of urethral damage compared to dermal healing.[3] Previous studies have shown EPO to be effective in the healing of dermal ulcers.[19] This effect of EPO is created by accelerating angiogenesis and reducing local inflammation. At the same time EPO stimulates the migration and proliferation of mesenchymal stem cells in the injury site. Growth factors such as endothelial growth factor, insulin-like growth factor, transforming growth factor, and fibroblast growth factor that are expressed from mesenchymal stem cells prevent the accumulation of excessive collagen type 3. Platelet-rich plasma with various growth factors has been shown to be effective in the prevention of fibrosis that develops following urethral injury.[20,21] The results of the current study also suggest that fibrosis was prevented with this same effect of EPO.

Recent clinical studies have determined high rates of ureteral stricture in patients undergoing chronic hemodialysis.[22] The authors stated that the EPO deficiency seen in chronic hemodialysis patients caused ureteral stricture. EPO is known to be one of the most important factors for recovery of the upper urinary tract system through the suppression of tubular epithelial cell apoptosis and enhanced tubular epithelial proliferation.[23]

To the best of our knowledge, the current study is the first in literature to have used EPO on the urethra. In the

group applied with high-dose EPO, fibrosis was seen at significantly low rates and in 3 of the 10 rats, no fibrosis was seen in normal urethral tissue at the end of 15 days. In the samples of both groups where EPO was used, fibrosis and inflammation were significantly less than in the control group. However, no statistically significant difference was determined between high-dose EPO and low-dose EPO in respect of fibrosis and inflammation. The stimulating effect of EPO on angiogenesis, which is one of the most important markers of wound healing and regarded as congestion of lamina propria in the histopathologic analysis, was clearly observed. In both EPO groups, congestion of vessels was observed at a significant rate in the lamina propria of almost all the samples. Although a significant difference was determined between the two EPO groups and the control group in respect of congestion, no statistically significant difference was determined between high-dose EPO and low-dose EPO.

In addition to being the main regulator of hematopoiesis, EPO is used as an important cytoprotective agent in organs such as the brain,[5-7] heart,[8] skin,[19] and upper urinary tract system.[23] However, there are no data showing the effect of the cytoprotective property of EPO in urethral trauma. The current study is the first in this field to show this effect. The main limitation of this study was that there were no long-term results as it was an animal experimental study.

A ready-to-use injector will make it easy to inject EPO into the urethral stricture area in future clinical trials. Local use will inhibit the systemic side-effects of EPO such as hemoglobin increase. A cost-effect analysis of medical treatment vs surgery management of urethral stricture can be debated in the future.

CONCLUSION

Erythropoietin can be considered a promising agent which could prevent fibrosis forming after urethral trauma and could accelerate urethral healing. There is therefore a clear need for further clinical studies with long-term results to be able to better evaluate this effect.

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EDITORIAL COMMENT

This is a nice contribution to the limited basic science knowledge we have in regards to urethral injury and urethral stricture disease. However, it is vital to understand the animal model

utilized in this study before any conclusions about clinical applicability in humans can be drawn.

The analogous clinical scenario in humans to the benchtop one depicted here in rats would be for a clinician to inject erythropoietin into the urethra immediately after a known (likely iatrogenic?) urethral injury so to prevent a possible urethral stricture. When is a urethra knowingly injured in a manner in which this scenario could be possible or even advisable? Almost never.

What researchers and clinicians alike are really looking for is a substance that can be injected into the urethra <u>after</u> urethrotomy for urethral stricture disease that will hasten scar reformation, keeping the urethral lumen open (>14F) and prevent the need for a urethroplasty.

There are a few candidates that have been investigated whose reported recurrence preventing mechanism is to modify (or prevent) collagen deposition after urethrotomy including, but not limited to, mitomycin,[1] triamcinolone,[2] and collagenase.[3] More recently, injectables that aim prevent recurrence by hastening endothelial and urethral regeneration, including Fat-derived Stem Cells,[4] platelet rich plasma[5] and even liquid buccal mucosa grafts[6] have also been described.

What do all of these injectables have in common? All of them are reported to provide clinical benefit. Why, then, are none of them being utilized routinely? — why are we still doing urethroplasties at all if they work so well? Most obvious is the need to follow these patients longer — and without well-designed RCTs, we'll never know to what degree the success can be attributed to the stricture characteristics, the urethrotomy or the injectable itself. Or perhaps these injectables just delay the recurrence (hence the initial enthusiasm) but don't prevent them (hence the lack of follow-up data or widespread adoption)?

However, more fundamentally, we likely don't have a winner yet because we don't understand how any of them work — and without an understanding of urethral stricture pathophysiology — how they form (nearly 60% of strictures are still labeled idiopathic!!), why they recur, and how they are related to other local and systemic disease processes[7] — we never will.

Add erythropoietin to the more recent amementarium of injectables that are hypothesized to help with local regeneration of tissue after urethrotomy for stricture disease. Though I believe regenerative injectables will be the key to eliminating the need for (most) urethroplasties, a specific modifiable target for the injectable within the stricture will need to be found first.

Bradley A. Erickson, MD, MS, FACS, Matthew D Grimes, MD, Associate Professor of Urology and Surgery University of Iowa, Carver College of Medicine

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AUTHOR REPLY

The most important problem of urethral wound healing is an increase in abnormal fibrosis because of imbalances in the healing processes. Healing of traumatic or idiopathic wounds in the urethra is known to be complicated and a longer process than dermal healing with sequential yet overlapping phases of hemostasis, inflammation, proliferation, and remodeling.[1]

The history of wound healing is old as the history of humankind. The oldest known medical record is a clay tablet that was written around 2200 BC.[2] To ensure that a wound healed properly and quickly, a wide range of practices and materials have been used throughout history. Furthermore, the ability to heal wounds quickly is among the most appealing of all technologies imagined by science fiction movies such as Transcendence (Pfister W, 2014) and Star Trek episodes. Such advanced technology is mostly based on the use of nanotechnology or nanomedicine.[3] Current research investigating ways of hastening the wound healing process continues to increase with advances in technology. The development of complex materials on a nanoscale (1−100 nm) provides a means capable of facilitating migration and proliferation through the controlled release of cytokines and growth factors such as endothelial growth factor, insulin-like growth factor, transforming growth factor and fibroblast growth factor, which form the complex signalling network that alters the growth, differentiation, and metabolism of targeted cells and prevents excessive abnormal collagen formation.[4,5]

Although the present study has some limitations as it was an animal study, it offers a window to the potential consequences of preventing excessive and abnormal fibrosis after urethral trauma. As stated in the editorial comments, longitudinal, larger, well-designed randomized controlled trials certainly need to be conducted, but it can be clearly stated that the present study is the spark, having secured a tipping point, because of the similarity of nano-technology methodology for the wound healing process. Many of the most significant developments begin with small imperfect steps that become strides towards improvement.

Muhammet Fatih Kilinc, Omer Gokhan Doluoglu, Department of Urology, Medical Science University, Ankara Training and Research Hospital, Ankara, Turkey

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