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ORIGINAL PAPER

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Biochemical analysis of urethral collagen content after tubularized incised plate urethroplasty: an experimental study in rabbits

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Abstract The aim of the present study was the biochemical analysis of tissue hydroxyproline levels in incised urethral plates in order to show the total collagen content after the Snodgrass operation in the hypospadiac rabbit model. The study comprised 21 male New Zealand rabbits, (2.2–2.4 kg). The animals were randomly allocated to three groups each containing seven rabbits as follows: group 1, the ventral urethra was completely excised and a model of hypospadias formed. A full-thickness incision was made on the distal dorsal urethra, a feeding tube was placed as an urethral catheter and both urethral wings were sutured ventrally. Group 2, inserting an iris knife into the urethra, the ventral wall was incised mimicking an urethrotome. Group 3 consisted of normal control rabbits to determine the basal tissue hydroxyproline level. A slight increase in the hydroxyproline level was observed in the ventral part of the urethral tissue compared to the dorsal part in both groups 1 and 2; however, these differences were not significant. After the Snodgrass operation in the rabbit model, no significant differences were observed in the hydroxyproline levels of the dorsal and ventral parts of the urethra or between these and of the controls. Further studies are required in order to determine the mechanism underlying urethral healing through normal re-epithelization without excess collagen deposition after incised urethral plate urethroplasty.

Keywords Hypospadias · Snodgrass · Urethra · Hydroxyproline · Collagen · Scar

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Introduction

Snodgrass (1994) described a tubularized incised plate urethroplasty (TIPU) technique for hypospadias repair. Recently, the TIPU operation has gained popularity for treating hypospadias. The key step in the operation is a deep longitudinal incision made through the midline of the whole urethral plate from the hypospadiac meatus distally. This widens the plate and allows it to be tubularized with no additional skin flap [1, 2]. Although TIPU is a widely applied procedure, the mechanism of healing is still obscure. Some authors consider that the incised plate heals by re-epithelization and regular dilatations are not necessary [3]; however, others think that urethral stricture formation is to be anticipated and that calibration after repair should be considered as an integral part of the technique [4, 5]. The connective tissue includes cells, mainly fibroblasts, elastic fibers and glycoproteins. The extracellular matrix plays important roles in tissue and organ development as well as in remodeling and wound repair. Excessive deposition of connective tissue is the pathological hallmark of fibrotic conditions, as for urethral stricture [6]. It is well known that total collagen, estimated via tissue hydroxyproline assessment, is of benefit in determining the developed of stricture in various tissues [6]. Thus, the aim of the present study was to investigate urethral healing using a biochemical analysis of tissue hydroxyproline levels of the urethral plate after TIPU in a rabbit model.

Materials and methods

Animals and experimental design

The study used 21 male New Zealand, white rabbits, 2.2–2.4 kg in weight. The rabbits were housed in a temperature and light controlled environment with ad libitum access to water and pellet food for 3 days before the study. All experimental procedures were approved by the local Animal Research Committee of the Faculty of Medicine. The rabbits were anaesthetized using 10 mg/kg ketamine hydrochloride and 4 mg/kg xylasine hydrochloride, introduced

intramuscularly, employing sterile techniques. The animals were randomly allocated into three groups each containing seven rabbits. In group 1, the ventral urethra was completely excised 1 cm from the meatus proximally and a hypospadiac penile model formed (Fig. 1). A full-thickness incision was made on the dorsal distal urethral plate; placing a 5 F feeding tube as a urethral catheter, both urethral wings were tubularized ventrally by a 7/0 polydioxanone running suture, and the penile skin approximated using 5/0 chromic catgut. In group 2, the ventral wall was incised while withdrawing an iris knife (mimicking an urethrotome) to see whether there is a healing difference between the dorsal and ventral urethral tissues. Group 3 consisted of normal control rabbits to determine the basal tissue hydroxyproline level. At 21 days postoperatively, the penises were harvested. All penises were bisected into ventral and dorsal parts and both samples were frozen in liquid nitrogen. Tissues were kept at −70°C until batch assessment of hydroxyproline levels.

Hydroxyproline assessment

As an indication of total collagen content, a quantitative determination of hydroxyproline was made using the spectrophotometric method of Woessner [7]. In this method, the hydroxyproline content is determined by a colorimetric reaction based on the oxidation of hydroxyproline to pyrolle, which reacts with *p*-dimethylaminobenzaldehyde to form a complex that is detected and quantified as a chromophore having an absorbance at 557 nm. The tissues are washed with cold saline solution, and then weighed on an electronic scale to give a sample of between 40–50 mg. Each sample was homogenized in 6 mol/l HCl. Homogenates were hydrolyzed for 3 h at 130°C. Freshly prepared 0.056 M chloramine T in 50% n-propanol and acetate and citrate buffer (pH 6.5) were added to the hydrolyzed samples and incubated at 25°C for 20 min. After adding 1 ml 3.15 mol/l perchloric acid, the mixture was incubated at room temperature for 5 min. Freshly prepared Ehrlich's aldehyde reagent in 2:1 n-propanol and perchloric acid was added to all samples and the tubes immersed in a water bath at 60 ± 2°C for 20 min. The tubes were then removed and cooled under running water. The absorbance of the colored product was determined at 557 nm on a spectrophotometer (Shimadzu, UV-120-02, Kyoto, Japan) and the amount of hydroxyproline was assessed by comparison with a standard curve (0–10 μg hydroxyproline). The collagen concentration was expressed as μg hydroxyproline/mg wet tissue weight.

Statistical evaluation

Statistical analyses were performed using SPSS 10.0 software for windows. Groups were compared using the Mann-Whitney U-test and paired Student's *t*-tests. A statistical value of $P < 0.05$ was considered significant.

Fig. 1 The experimental hypospadias model in rabbits

Results

Tissue hydroxyproline levels from the dorsal and the ventral parts of the urethra in the study and control groups are given in Table 1. When the ventral parts of the bisected penises (7.75 μg/mg ± 1.43) were compared with their dorsal (6.74 μg/mg ± 0.90) counterparts in group 1 (Snodgrass), a slight increase in the hydroxyproline levels was observed. Similar increases in the hydroxyproline levels were found in the ventral parts (7.40 μg/mg ± 1.06) when compared with those of the dorsal parts (6.44 μg/mg ± 1.24) in group 2 (ventral incision). However, these differences were not significant. When the ventral and the dorsal parts of the urethral tissues of groups 1 and 2 were compared with the ventral and the dorsal parts of normal controls, again no significant differences were found.

Discussion

Snodgrass et al. [8] state that the healing processes described in the literature show a parallel to the clinical outcome of TIPU, which most often heals without evidence of scarring. Since relaxing incisions resembled optical urethrotome applied for urethral strictures, the initial opinion is that it may also result in stricture formation; however incisions on the dorsal urethral plate are seen to heal without significant scarring [9]. In our previous experimental histological study, the urethral plate also healed by re-epithelization without significant subepithelial scarring [10]. Bluestein et al. [11] and Lopes et al. [12] in their experimental studies on dogs and pigs report similar findings. An increase in the vascularity of the hypospadiac penis was reported by Baskin et al. [13]. Erol et al. [14] point to the fact that the major difference between the hypospadiac and the normal penis is the extensive vascularity of the abortive urethral spongiosum and glans in congenital hypospadiac penis. This ultrastructure of the hypospadiac penis may help to explain the success of healing with no scarring after tubularized incised plate urethroplasty [15]. Nevertheless, all of the experimental models involve normal animals made hypospadiac. This rich vascularity of the dorsal plate is not present in our hypospadiac rabbit

Table 1 Tissue hydroxyproline levels from dorsal and ventral sections of the urethra in study and control groups. All values are given as mean ± standard deviation (μg/mg wet weight, $n = 7$ for each group)

	Dorsal	Ventral	*P*
Group 1 (Snodgrass operation)	6.74 ± 0.90	7.75 ± 1.43	0.194
Group 2 (ventral incision)	6.44 ± 1.24	7.40 ± 1.06	0.159
Group 3 (control)	6.55 ± 0.53	6.42 ± 0.75	0.695

model and also the dog and pig models of other authors. Interestingly, all of the above animal models show that the blood supply in the normal dorsal urethra in the models made hypospadiac could be adequate for recovering the full thickness incision without scarring [10, 11, 12]. Several factors may contribute to the success or failure of urethroplasty. They include ischemia, the patient's age, aetiology and length of the stricture, as well as, according to some authors, a previous urethrotomy [16].

In the present study, although a slight increase in the hydroxyproline levels was observed in the ventral urethral tissues of the rabbits in relation to dorsal sections in groups 1 and 2, these differences were not statistically significant. In addition, in group 1, no significant difference was found in the hydroxyproline level of the dorsal urethra, which is the incised urethral plate, in relation to that of the controls. Baskin et al. [17] report the biochemical characterization and quantitation of the collagenous components of urethral tissue and state that total collagen content, as determined by hydroxyproline analysis, revealed no significant differences between the control and the study groups. Although no difference was detected in total collagen content of strictured urethral tissue, significant alterations in the type III:I collagen ratio has been reported by the same authors. We agree that the main change in collagen metabolism in urethral stricture disease is probably a decreased ratio of collagen III:I. Unfortunately, in our study the quantitation of the type I and type III collagens could not be assessed. Da Silva et al. [6] report that the bulbar urethra shows the lowest concentration of total collagen, while the glandular urethra has the highest content. They also state that glycosaminoglycans and collagen are major components of the extracellular matrix, and that they play key roles in the urethral strictures. These authors note significant increases in total collagen content and this change is more evident when the glycosaminoglycans to collagen ratio are considered [18].

Previous studies have shown that the bladder urothelium has the ability to regenerate if the proper extracellular matrix scaffold is present [19, 20]. Baskin et al. [21], report growth factors in bladder wound healing and conclude that keratinocyte growth factor and transforming growth factor-alpha are transcriptionally upregulated early in bladder wound healing when urothelial proliferation is necessary to resurface damaged tissue. It has been reported that epidermal growth factor receptor regulates normal urothelial regeneration [22, 23]. We are of the opinion that the same processes could occur during the healing of the incised urethral plate. We believe that the rate and the quality of urethral wound healing could be affected more by growth factors than the extensive vascularity of the urethral plate.

In conclusion, after the Snodgrass operation in the rabbit model, no significant differences were observed in the hydroxyproline levels of the dorsal and ventral parts of the urethra and between those and of their controls. We are of the opinion that further studies, e.g. assessment of growth factors, are required before the mechanism underlying urethral healing, through normal re-epithelization without excess collagen deposition after incised urethral plate urethroplasty, is revealed.

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