

Study of Collagen in the Urethral Plate of Children with Hypospadias

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Abstract:

Aims & Objectives – The study was performed to quantify the amount of Collagen in the Urethral Plate of Children with Hypospadias. Masson trichrome stain (MT) was compared with picrosirius red stain (PSR) and their outcomes.

Materials & Methods – The study was performed in 25 children (age 1 year to 12 years). After TIP procedure, biopsy was taken from the Urethral plate. The tissue was subjected to MT and PSR staining, and correlated with post-operative status at 15 days, 1 month and 3 months.

Result:- MT score versus PSR score was correlated. MT score ranged from 1- to 3+ and PSR score ranged from 1- to 3+. The post-operative outcomes at 2 week, 01 month & 03 months had insignificant p-value with PSR score. The position of neourethra and Residual Chordee were found to be significant with p-value = 0.05. The sensitivity of PSR scores with MT score as gold standard was 56.25% with 95% CI of 29.88% to 80.25%.

Conclusion: - Residual chordee was present more in proximal hypospadias as found by both MT and PSR leading to poorer outcome. Distal hypospadias had good result with both MT & PSR stains using fluorescent polarizing microscopy.

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I. Introduction

Hypospadias is one of the most common congenital defects affecting the external male genitalia^{1,2}. The incidence is 1 in 250 newborns or roughly 1 out of 125 live male births, although its incidence seems to be increasing^{3,4}.

Hypospadias is defined as an insufficient development of the urethral fold and the ventral foreskin, with or without penile curvature. The urethral opening is located more proximally anywhere between the tip of the penis and the perineum^{1,4}.

Urethral plate is the strip of tissue that extends distally from the hypospadiac meatus to near the tip of the glans. There is no literature comparing differences between histology of urethral plates of distal and proximal hypospadias using Masson Trichome stain to quantify collagen and compare with Picrosirius red stain. Comparison of both the stains with post-operative outcomes was obtained.

II. Material And Methods

Twenty-five cases hypospadias (11 proximal & 14 distal) in children aged 1 to 12 years were included in this study over a period of 1 year (2015-2016). Detailed history and thorough clinical examination was done and recorded. All these patients underwent single stage surgery (Snodgrass urethroplasty). At the time of midline incision of urethral plate, urethral plate was stretched laterally & a strip of urethral plate tissue of at least 2 mm × 2 mm × 0.5 mm (length × width × thickness) was taken and sent for histopathological examination.

Clinical details

Parameters like name, age, sex, weight, date of admission/ operation/ discharge, detailed clinical history & examination, history of previous surgery, diagnosis, any associated significant disease, routine investigation, USG, urine c/s report were noted.

Operative procedure

Tubularisation of incised plate (TIP) urethroplasty procedure was done in every case. All procedures were either performed or supervised by at least one of the senior surgeons following identical surgical steps.

Tissue harvesting

At the time of midline incision of urethral plate, stay sutures with vicryl 5-0 were taken on bilateral sides of urethral plate at urethral meatus. Urethral plate was stretched laterally & a strip of urethral plate tissue of at least

2mm × 2 mm × 0.5mm (length × width × thickness) was taken and sent for histopathological examination in 10% formalin sample container.

Gross examination

All the urethral plate specimens were fixed in 10% neutral buffered formalin for appropriate time till the tissue was well fixed and all were processed.

Firstly all samples were stained with Hematoxylin & Eosin, examined and results were recorded. The stained slides were examined using a 40 × objective and 10 × eyepiece for sectioned collagen fiber bundle. In H&E stain, collagen fiber bundles were seen as thick and irregular pink colored structures. Then, Masson’s Trichome staining done and samples were examined especially for collagen fibers and results were recorded. With MT stain, collagen fiber was seen as blue stained irregular linear structures. According to amount of collagen fiber bundles in samples, visual interpretations were done by single pathologist and urethral plate specimen graded as following:-

Table 1: Grading of collagen fibers in Mason Trichome stain (in 400 × magnification)

1-	Sparsely arranged collagen fibers
1+	Very less densely arranged collagen fibers
2+	Less densely arranged collagen fibers
3+	Densely arranged collagen fibers

Afterwards, the urethral plate tissue was subjected for staining with Picrosirius red (PSR) for determining the type of collagen by polarising and fluorescent microscopy. With Picrosirius Red, collagen type I shows thick, strongly birefringent, yellow or red fibres. Collagen type III appears as thin, weakly birefringent greenish fibres that could be identified as reticulin fibres.

Table 2 – PSR score

Collagen type 1 & 3 quantity in urethral plate as seen in polarizing microscope with picrosirius red stain (in 400 × magnification)	PSR SCORE
Type 1>>3	2-
Type 1>3	1-
Type 1=3	1
Type 1<3	1+
Type 1<<3	2+

The Masson Trichome (MT) was considered as gold standard and Picrosirius Red (PSR) was compared for determining its efficacy as a predictive tool of prognosis in post-operative hypospadias.

Postoperative management and follow-up:

Length of stay and postoperative complications were registered. After the discharge from hospital the patients were followed at 2 weeks, 1 month and 3 month intervals in OPD & follow up clinics.

The patients were being assessed on the basis of:

- a) HPE report
- b) Neourethra assessment by calibration with standardized infant feeding tube
- c) Clinical condition , cosmesis & residual chordee
- d) Functional outcome.

Follow-up checks for results as well as for any complications were performed on all children at 2 weeks, 1 month and 3 months intervals. Those examinations included history, observation of the urinary stream, thorough inspection of the penis & urinalysis.

Neourethral calibration was done at 1st and 3rd months of follow-up. Calibration was performed at an OPD, by calibrating the urethral meatus according to the age of the child. The meatus of a boy under 1 year of age should accept a 5-Fr feeding tube; between 1 and 6 years this increases to 8 Fr. Neourethra assessed and grade as good or poor. Result was labeled as poor when there was presence of meatal stenosis, urethral stricture or any urethro-cutaneous fistula.

Residual chordee was assessed as present and absent. Significant chordee was clinically defined as curvature greater than 20°; only then chordee correction was done. Post –op assessment of chordee was done, if it was more than 20° then it was labeled as residual chordee present.

Cosmesis was assessed at the end of 3rd month and graded as good and poor. Cosmetic appearance was judged by the location of the meatus and the skin cover and the absence of any residual chordee. The condition of the ventral skin and the presence of any excess dorsal skin were also assessed.

Final outcome was graded as good, moderate and poor. Success was defined as the presence of an anatomically positioned vertical slit-like meatus, a normal urinary stream, and a satisfactory cosmetic appearance resembling a circumcised penis.

The mean results were recorded for analysis.

Statistical methods

Descriptive statistics like mean, median and proportion were used to describe the study result and appropriate statistical test were applied. SPSS software (SPSS 17, Chicago, Illinois, USA) was used for statistical analysis. Student's t-test was performed for the comparison of mean value. Nominal variables were compared using Pearson's Chi-square test or Fisher's exact test. Statistical significance was defined as a p-value $p \leq 0.05$ (two-sided).

III. Result

Our study was a prospective observational study which was carried out on 25 patients of hypospadias (11 cases of proximal & 14 cases of distal hypospadias) which presented to our department. All patients were new cases under 12 years of age. Patients underwent preoperative workup for anesthesia fitness and admission demographic, clinical, laboratory investigations, operative and pathological data were entered according to the proforma approved by the IRB and Ethics committee.

All patients had signed a special consent and were provided with a patient information sheet. After the discharge from hospital the patients were followed at 2 weeks, 1 month and 3 month intervals. The data collected for each patient was entered into Microsoft Excel and was analyzed statistically using SPSS 17 software.

The detailed histology of collagen as depicted in Masson Trichome stain (Figure 1) and Picrosirius Red stain (Figure 2)

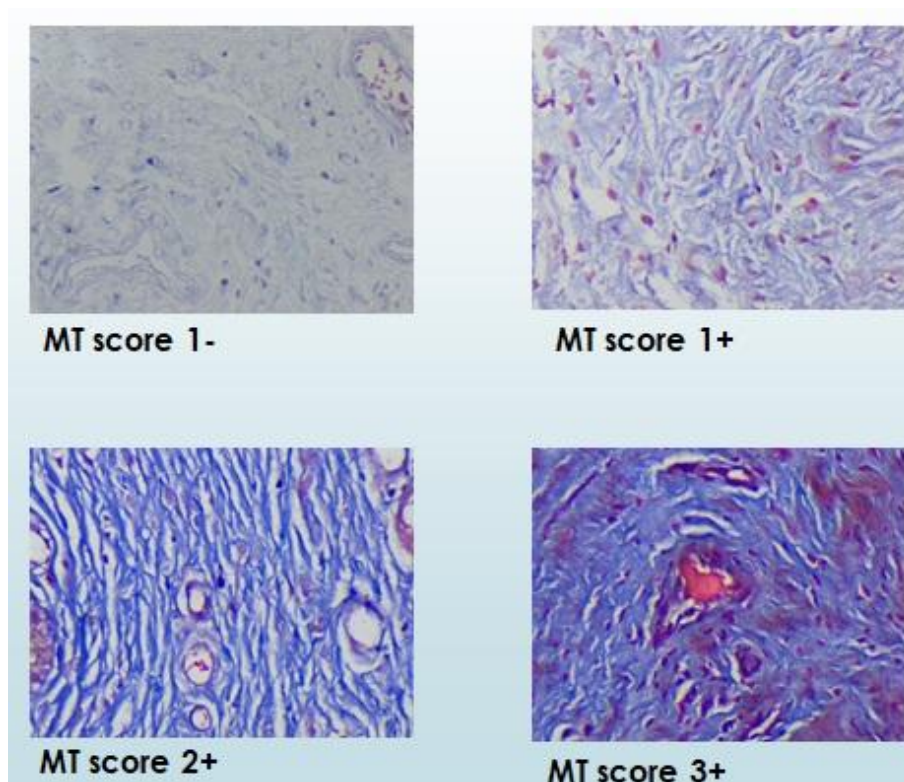


Figure 1: collagen fibers in Mason Trichome stain (in 400 × magnification)

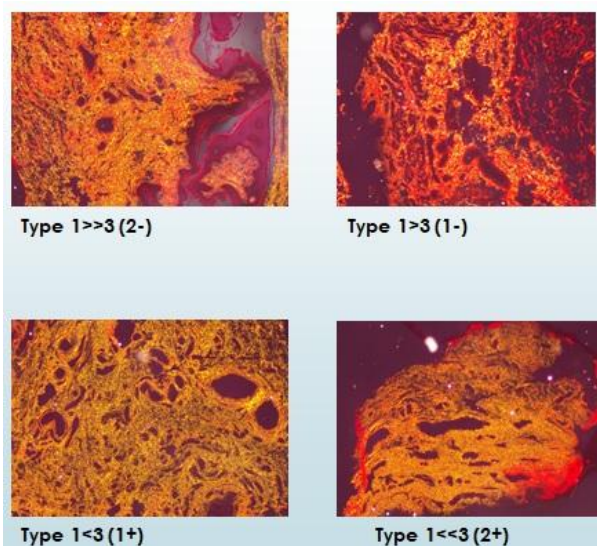


Figure 2: Collagen type 1 & 3 quantity in urethral plate as seen in polarizing microscope with picosirius red stain (in 400 × magnification)

The following observations were made comparing MT score with PSR score (table 3)

Table 3- comparing MT score with PSR score

		PSR score						Total	Chi-square value	p-value
		1	1-	1+	2-	2+	3-			
MT score	1-	0	0	1	1	0	0	2	21.161	0.132
	1+	1	6	2	0	4	1			
	2+	2	0	0	3	1	1			
	3+	1	0	1	0	0	0			
Total		4	6	4	4	5	2	25		

Post-operative outcomes at 2 weeks, 1 month and 3 months were correlated with PSR score (table 4, 5 and 6).

Table 4- Post-operative outcomes at 2 weeks correlated with PSR score

		PSR score						Total	Chi-square value	p-value
		1	1-	1+	2-	2+	3-			
complication after 2 wk	fistula	1	0	0	0	0	0	1	5.469	0.361
	nil	3	6	4	4	5	2			
Total		4	6	4	4	5	2	25		

Table 5- Post-operative outcomes at 1 month correlated with PSR score

		PSR score						Total	Chi-square value	p-value
		1	1-	1+	2-	2+	3-			
complication after 1 month	fistula	1	0	1	0	1	0	3	19.912	0.175
	meatal stenosis	0	0	0	2	0	0			
	nil	2	6	3	2	4	2			
	res chordee	1	0	0	0	0	0			
Total		4	6	4	4	5	2	25		

Table 6 - Post-operative outcomes at 2 weeks, 1 month and 3 months were correlated with PSR score

		PSR score						Total	Chi-square value	p-value
		1	1-	1+	2-	2+	3-			
comp'n after 3 m	breakdown	1	0	0	0	0	0	1	30.97	0.417
	fistula	1	0	1	1	1	1			

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	fistula+stenosis	0	0	0	1	0	0	1		
	meatal stenosis	0	1	0	1	0	0	2		
	nil	0	5	3	1	4	1	14		
	res chordee+ stricture	1	0	0	0	0	0	1		
	stricture	1	0	0	0	0	0	1		
Total		4	6	4	4	5	2	25		

Position of neourethra and residual chordee were determined with PSR score in Table 7 and 8.

Table 7- Position of neourethra correlated with PSR score

		PSR score						Total	Chi-square value	p-value
		1	1-	1+	2-	2+	3-			
Neourethra	fistula	2	0	1	1	1	1	6	24.901	0.051
	good	0	5	3	1	4	1	14		
	meatal stenosis	0	1	0	2	0	0	3		
	stricture	2	0	0	0	0	0	2		
Total		4	6	4	4	5	2	25		

Table 8 – Residual chordee correlated with PSR score

		PSR score						Total	Chi-square value	p-value
		1	1-	1+	2-	2+	3-			
Res. Chordee	absent (0)	1	6	1	3	4	2	17	10.983	0.052
	present (1)	3	0	3	1	1	0	8		
Total		4	6	4	4	5	2	25		

The final outcome was graded into 3 categories as based on PSR score (table 9) with straight comparison of both MT and PSR scoring system in table 10.

Table 9- The final outcome was graded into 3 categories as based on PSR score

		PSR score						Total	Chi-square value	p-value
		1	1-	1+	2-	2+	3-			
final outcome	good (2)	0	5	1	1	4	1	12	16.424	0.088
	moderate (1)	1	1	2	0	0	0	4		
	poor(0)	3	0	1	3	1	1	9		
Total		4	6	4	4	5	2	25		

Table 10 - Straight comparison of both MT and PSR scoring system

		PSR score		Total
		1	1+	
MT score	1-	1	1	2
	1+	8	6	14
	2+	6	1	7
	3+	1	1	2
Total		16	9	25

The final statistical analysis revealed sensitivity and positive predictive value of 56.25% (table 11).

Table 11- The final statistical analysis

Statistic	Value	95% CI
Sensitivity	56.25%	29.88% to 80.25%
Specificity	22.22%	2.81% to 60.01%
Positive Likelihood Ratio	0.72	0.41 to 1.26
Negative Likelihood Ratio	1.97	0.51 to 7.54
Disease prevalence	64.00%	42.52% to 82.03%
Positive Predictive Value	56.25%	42.45% to 69.15%
Negative Predictive Value	22.22%	6.94% to 52.25%

IV. Discussion

Over the last few decades, hypospadias surgery has changed after the identification of the urethral plate as an anatomical entity. The most important is the preservation of the urethral plate and second is the in situ tubularisation of the plate¹³. The longitudinal midline incision in urethral plate helps to make tension free tubularisation to form a neourethra of an adequate size. Baskin et al in their histological study of urethral plate found submucosal layer of plate is highly vascularised with good nerve supply. This property of healing with re-epithelialisation decreases the chances of urethral stricture resulting in good urethral stream^{14,15,16}.

There are very limited studies done on histopathology of urethral plate in human hypospadias. Yucel et al reviewed the literature regarding the histological findings and observed that there was no difference in vascularity between Hypospadiac and normal penis¹⁷. Snodgrass et al carried out a histological study using subepithelial biopsy specimens of the urethral plate in boys undergoing tubularized incised plate urethroplasty and reported that the histological features consisted of well-vascularized connective tissue, composed of collagen and smooth muscle interspersed with blood vessel and nerves, without evidence of fibrous bands or dysplastic tissue¹⁸. It has been found that the most of the complications of surgery i.e. urethro-cutaneous fistula (80%) occurs at coronal level¹⁹. This may be due to decreased vascularity as frenular artery is constantly missing in hypospadiac penis²⁰.

Erol et al studied on urethral plate of newborn penis with hypospadias stained with Hematoxylin and Eosin, Immunohistochemical staining, Masson's trichrome and Picro-Sirius Red (PSR) to localize collagen⁹. They found that the tunica albuginea stained intensely for type I and III collagen which explains the lower complication rate with onlay flaps than with tube flaps.

Hayashi et al studied on urethral plate characterization observed by transmission electron microscopy (TEM)²¹. They observed the distinctive feature of the vessels of the cavernous sinus, intertwining and massing, in the capillary vessels of the tissue by Hematoxylin& Eosin staining and immunohistochemical staining, which implies that the origin of the tissue beneath the urethral plate is the same as the corpus spongiosum penis. Excess deposition of collagen subtype I occurs in the maturation phase of scar formation, resulting in the formation of large, stiff bands of fibrils, which might consequently cause less tumescence in patients with scar formation or fibrosis of the corpus cavernosum penis. Collagen subtype III is also found in the distensible elastic tissues (e.g. lung, liver, spleen, kidney) in the presence of collagen subtype I. It is also the first deposited collagen in the subtype III to subtype I.

Baskin et al proposed that the total collagen amount in the tissue of urethral stricture does not change, but rather its subtype ratio changes in favor of collagen type III²². Collagen subtype IV, a component of the basement membrane, is believed to be secreted by endothelial cells and might be related to the microangiopathy of diabetes mellitus. Collagen has been thought to be the main component of the fibrous skeleton of the corpus cavernosum penis and is ubiquitous within the erectile tissues of the human penis.

Akan et al concluded that knowing the absolute counts of blood vessels and collagen fibre bundles are important rather than relative proportions of sectioned blood vessels and collagen fibre bundles on histological differences between ventral and dorsal skin of hypospadiac penis²³.

In the study done by da Silva et al on age related structural changes of the urethral plate in hypospadias, they characterized extracellular matrix structure with various stains [Hematoxylin& Eosin, Masson's trichrome, Weigert's resorcin-fuchsin and Picro-Sirius red(PSR)]²⁴. They concluded that total collagen concentration in urethral plates increased significantly with aging which may have a role in urethral healing following hypospadias repair.

Study done by Lopes et al in animal models for histological analysis of urethral healing after hypospadias repair suggested that measured outcomes were degree of epithelial healing and quantification of collagen deposition assessed with Masson Trichrome stain²⁵. It could be expected that a prolonged inflammatory

reaction and excess collagen deposition would precede scar formation but neither was demonstrated in their study. Their study has several limitations, as do all animal studies. As in the human hypospadiac penis the depth of the urethral plate is greater, allowing for a deeper incision before tubularisation. The results of their study support the conclusion that dorsal incision of the urethra heals by rapid urothelial ingrowth in the absence of excess collagen deposition or scar formation.

In another study in our institution we had determined the differences in amount of collagen tissue in urethral plate of proximal and distal hypospadias. We had also further correlated the postoperative outcomes in proximal and distal hypospadias as determined by the histological picture of urethral plate as per collagen content.

In this study the usage of both this stain MT and PSR (by polarizing fluorescent microscopy) helped determining the subtypes of collagen in a definitive manner. This helped correlating the amount and type of collagen with subsequent post-operative outcomes. A larger patient sample size will be required in future studies to develop this histological tool with children with hypospadias.

V. Conclusion

This present study had concluded that presence of residual chordee in proximal hypospadias (confirmed by MT & PSR score both) led to poorer outcomes. Distal hypospadias had better result as found by MT & PSR stains differentiating type 1 and type 3 collagen. Type 1 collagen had better outcome enabling us to prognosticate such children for long term. This illustrates the importance of basic histological studies in predicting surgical outcomes in children with hypospadias.

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