Evaluation of the Intravesical Ureters after Failed Endoscopic Treatment of Vesicoureteral Reflux with Dextranomer/hyaluronic Acid in Children via Light and Transmission Electron Microscopic Analysis. A Matched Case-Control Study

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Introduction: The cytokine profile and the ultrastructural changes of refluxing ureterovesical junctions(UVJs) of children treated with failed dextranomer/hyaluronic-acid (Dx/HA) injections were investigated using immunohistochemical methods and transmission electron microscopy(TEM).

Patients and Methods: Eighteen children who had undergone injection for reflux were included the study. The smooth muscle arrangement of the ureteral wall, transforming growth factor-β (TGF-□1),vascular-endothelial-growth factor (VEGF) and CD34 were evaluated immunohistochemically, and the results were compared with 10 age-matched autopsy specimens as controls. The ultrastructural evaluation and morphological description was made semi-quantitatively and compared with published data.

Result: Four of the patients (22%) were male, and 14 (78%) were female. The mean age of the patients was 105.4 ± 44.5(48-184) months. There was no correlation between the vesicoureteral reflux (VUR) grade and age ($P = 0.85$). The mean VEGF and CD34 scores were 16.2 ± 9.6 (0-90) cells per HPF and 10.2 ± 3.5 (4-16) vessels per HPF in ureters with reflux; these values were 60.6±16.4 (32-84) cells per HPF and 17.8 ± 4.1 (12-24) vessels per HPF in the control group. The amount of VEGF and CD34 were significantly decreased in patients compared with the control group ($P < 0.001$, $P < 0.001$).The TGF-β1 levels were significantly higher in patients with VUR compared with the control group (34.2 ± 19.9 vs 5.0±1.9; P=0.001).The amount of VEGF, CD34, and TGF-β1 were not correlated with the grade of reflux ($P = 0.26$, $P = 0.94$, and $P = 0.42$, respectively). Ultrastructural changes in the muscle cells were observed in all the VUR specimens (Grade II-IV).

Conclusion: Refluxing ureters exhibited immune-histopathological abnormalities and ultrastructural changes of the muscle cells in all VUR specimens in the ureterovesical junctions of children treated with failed Dx/HA injections for reflux.

Keywords: dextranomer/hyaluronic acid; transmission electron microscopy; ureterovesical junctions; vesicoureteral reflux

INTRODUCTION

Vesicoureteral reflux (VUR) is characterized by the retrograde flow of urine from the bladder into the upper urinary tract because of an anatomic and/or functional disorder. Moderate to severe reflux is responsible for potentially serious consequences such as renal scarring, hypertension, and renal failure. Antibiotic prophylaxis and surgical management by ureteroneocystostomy have been the traditional treatments of choice for VUR since the 1970s[1-3] . In the early 1980s, a less invasive technique of endoscopic injections (EI) of bulking agents was first described by Matouschek et al.[4] This technique became popular after O'Donnell and Puri(1986) published their successful initial report on the endoscopic correction of primary reflux[4-8] .
Dextranomer/hyaluronic acid(Dx/HA) is formed from cross-linked dextranomer microspheres suspended in a carrier gel of stabilized sodium hyaluronate. This finding was first described by Stenberg and Lackgren in 1995[9]. The Food and Drug Administration (FDA) approved this material for use in children with primary grade I-IV VUR in 2001.Since then, several clinical investigations have been published with high success rates that range from 68% to 89% [8,10-12]) . Very rare complications have been reported[13-14]. The histopathological effects of Dx/HA on the intravesical ureters have been investigated by only a limited number of studies[15-19] .There are no data in the literature regarding the evaluation of ultrastructural changes in the intravesical ureters by transmission electron microscopy (TEM) in patients with failed EI. To our knowledge, this is the first study to evaluate the ultrastructural changes using TEM in the ureterovesical junctions(UVJ) of children treated with failed Dx/HA injections for reflux.
Additionally, cytokines play significant roles in VUR

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pathophysiology. TGF-β1 is involved in many cellular functions, including cell growth, proliferation, differentiation and apoptosis. Increased levels of this cytokine may induce apoptosis in smooth muscle cells, leading to contractile dysfunction and structural abnormalities [20] .VEGF is a signal protein and produced by muscle and endothelial cells to stimulate vasculogenesis and angiogenesis, is important for regulating tissue growth, nerve coordination and gap junction metabolism. VEGF is typically less abundant in poorly developed UVJs[20]. CD34 is an endothelial antigen whose function is unknown. The anti-CD34 antibody is often used as a marker to determine microvessel density, which has been previously studied to determine the role of hypoxia in pathogenesis [21] .

In this study, cytokine profile, including the transforming-growth-factor-β1 (TGF-β1), vascular-endothelial-growth factor (VEGF), and CD34 of the distal ureters were evaluated quantitatively by immunohistochemical methods, and the ultrastructural evaluation and morphological description was made semi-quantitatively and compared with published data.

PATIENTS AND METHODS

Between January 2013 and August 2014, 18 children who underwent ureteroneocystostomy for VUR following failed injection therapy with Dx/HA were included this prospective a case control study. The indications for surgery in the study group were reflux persistency or greater reflux on voiding cystourethrography and recurrent upper urinary tract infections. After obtaining informed consent from all patients' families, urodynamic studies were performed in all patients to rule out neurogenic bladder and voiding dysfunction before the operation. Patients who had a history of previous open reimplantation for reflux or any other ureteral diseases were excluded from the study. After obtaining the necessary approvals from the local ethics committee (approval number-March 1,2012;6/4), the parents or legal guardians of the patients were informed that the clinical and laboratory data would be used for scientific purposes, and written consent was obtained.

Histopathological and ultrastructural evaluations

28 distal intravesical ureters were dissected sharply without cauterization during ureteroneocystostomies for histopathological and ultrastructural evaluations. Each sample contained an intramural portion of the ureter with the ureteric orifice. The size of the specimen approximately 15 mm. Depending on the order of excision, the preparations were placed in vials that contained formaldehyde for histopathological evaluation or vials that contained a 5% glutaraldehyde solution for ultrastructural evaluation with an electron microscope. All the vials were numbered and evaluated by a single pathologist and histologist. The ureterovesical junctions of 5 age-matched autopsy specimens was done Faculty of Medicine, University of Çukurova Between January 2013 and August 2014 without history or evidence of any urological disease (2 with respiratory distress syndrome,1 with aspiration pneumonia and 2 who experienced sudden death) served as the control group and were used only for histopathologic and immunohistochemical evaluations.

Electron microscopic examination

The specimens of the distal intravesical ureteric segments for electron microscopic examination were fixed for 4 hours with 5% glutaraldehyde in Millonig phosphate buffer at pH 7.4 and post-fixed with 1% osmium tetroxide in the same phosphate buffer for 2 hours at 4 °C. The samples were dehydrated in a graded series of ethanol and embedded in araldite. Semi-thin sections were taken with Reichert Ultracut-S ultramicrotome and stained with toluidine-blue, and the appropriate areas for electron microscopic observation were determined. Thin sections were taken from the selected areas and stained with uranyl acetate and lead citrate. They were examined with a Jeol JEM 1400 transmission electron microscope. The ultrastructural evaluation and morphological description was made semi-quantitatively.

Light microscopy study

For the histopathological evaluation, formaldehyde-fixed, paraffin-embedded tissues were cut transversely at a 5-µm thickness. Hematoxylin and eosin (HE) stained slides were examined under light microscopy (Nikon-E600,Tokyo,Japan).The smooth muscle arrangement of the ureteral wall was scored as Oswald et al.[23] described in their study that was based on the absence of a muscular coat, the replacement of muscle fibers with fibrotic tissue, and the enhancement of interstitial collagen: Score 0-absent, 1-mild (≤ 25%), 2-moderate (26-50%), 3-severe (51-75%), and 4-extremely severe (> 75%).The results were compared with the control group.

Immunohistochemical examination

Immunohistochemistry was performed on formalin-fixed, paraffin–embedded 5 µm thick tissue sections using a manual streptavidin-biotin complex immunoperoxidase procedure with antibodies against human VEGF (monoclonal mouse, Dako,M7273; Denmark), TGF-□1 (polyclonal rabbit,Santa-Cruz,sc-146), and CD34 (monoclonal mouse,DAKO,M7165, Denmark). For all the antibodies tested, antigen retrieval treatment for 15 min in 0.01 M citrate buffer solution (pH 6.0) using a microwave oven was performed, and the immune complexes were then visualized by AEC. The slides were counterstained with Mayer's hematoxylin and mounted.The positive controls were angiosarcoma, tonsil, and, hemangioma. The negative controls were obtained by omitting the primary antibody. All VEGF and TGF-β1-positive cells were counted from 10 randomly selected high-power fields (HPFs) at 400x magnification. The urothelial and intraluminal cells were not counted. Microvessel densities were evaluated by counting positively stained endothelial cells or cell clusters in 10 randomly selected HPFs. The results were compared with the control group and between each grade.

Statistical analysis

A data analysis was performed using SPSS software, version 15 (SPSS,Inc.,Chicago,IL). The chi-square test, T-test and one-way analysis of variance(ANOVA) were used for analysis. In all the tests, the statistical significance level was set at $P<0.05$.

RESULTS

Of the patients, 4 (22%) were male and 14 (78%) were female. The mean age of the patients was 105.4 ± 44.5(48-184) months. The reflux was grade II in 6, grade III in 12, and grade IV in 10 ureters, according to the International Reflux Study[22] (**Table 1**). None of

Table 1. Sample distribution by reflux grade.

Reflux Grade	Number of ureteral units	
	Right	Left
I	-	-
II	4	2
III	4	8
IV	2	8
V	-	-
Total	10	18

Table 2. Cytokine profile of the patients.

	Grade (n)			
	II(n=6)	III(n=12)	IV(n=10)	*P*
VEGF	14.0 ± 3.0 (6-26)	4.8 ± 4.3 (0-10)	31.2 ± 35.5 (2-90)	0.26
CD34	14.8 ± 2.0 (12-16)	10.1 ± 2.9 (6-14)	8.0 ± 3.1 (4-12)	0.94
TGF-β1	33.3 ± 15.2 (20-50)	34.6 ± 24.7 (8-60)	34.4 ± 20.1 (12-60)	0.42
SMS	1.6 ± 0.5 (1-2)	1.0 ± 0.6 (0-2)	1.2 ± 1.3 (0-3)	0.86

* VEGF = vascular-endothelial-growth factor
*TGF-β1= transforming growth factor-β1
*SMS = Smooth muscles scores

the patients had grade V VUR. There was no correlation between the VUR grade and age ($P = 0.85$). 10 patients had bilateral VUR, and 8 had unilateral VUR. The mean injected volume for each ureter was 1.0 cc, and the mean time from EI to surgical intervention ranged from 3 to 10.2 ± 7.9 months. The mean injection number was 1,07 ± 0,26 times. No preoperative or postoperative complications were observed.

Electron microscopic evaluation

The surface urothelium, lamina propria, and adventitia were observed as normal in all grade VUR specimens (**Figure 1A**). Ultrastructural changes of the muscle cells were observed in all the VUR specimens (Grade II-IV). Intercellular edema and increased cytoplasmic density of some smooth muscle cells were observed in all the specimens (**Figure 1B, 2A, 2B**). Heterochromatins clumping in the nucleus and perinuclear cisternae enlargement were observed in grade III-IV VUR specimens (Figure 1B, 2A). Swollen endoplasmic reticulum cisternae and mitochondria, cristae disorganization in mitochondria, and vacuoles that include membranous whorl structures in some areas and empty spaces that characterize edema in the cytoplasm of the smooth muscle cells were prominent in all the VUR specimens (Grade II-IV) (**Figure 1A, 2A, 2B**). The degree of degeneration was similar in patients with the same grade VUR who were different ages.

Histopathology

Dx/HA material was located in the adventitia in 22 (78.6%) cases and in the muscle fibers in 6 (21.4%) cases. A fibrous pseudocapsule surrounding the Dx/HA material was present in only two (14.3%) cases, which were located in the muscle fibers. A giant cell reaction was rapid in 26 (92.9%) of the 28 cases. In three (21.4%) cases, eosinophilic infiltration was increased compared to the other cases. No calcification or rapid inflammation was observed. In most of the VUR cases, the smooth muscle coat was disorganized and widely absent compared to the control group. Collagen and edema was replaced instead of smooth muscle. The mean smooth muscle scores in grade II to IV VUR were 1.6 ± 0.5 (1-2), 1.0 ± 0.6 (0-2), and 1.0 ± 1.4 (0-3), respectively. No significant correlation was found between the reflux grade and the smooth muscle disarrangement score ($P = 0.86$). When we compared the results with the control group, the difference was significant ($P < 0.001$). There was no sign of inflammation, operative injury or cautery artifact in the specimens.

Immunohistochemistry

There was a significant difference in the amount of cytokines between the patients and the controls (**Figure 3**). The mean VEGF and CD34 scores were 16.2 ± 9.6 (0-90) cells per HPF and 10.2 ± 3.5(4-16) vessels per HPF in ureters with reflux; these values were 60.6 ± 16.4 (32-84) cells per HPF and 17.8 ± 4.1 (12-24) vessels per HPF in the control group. The amount of VEGF and CD34 were significantly decreased in patients com-

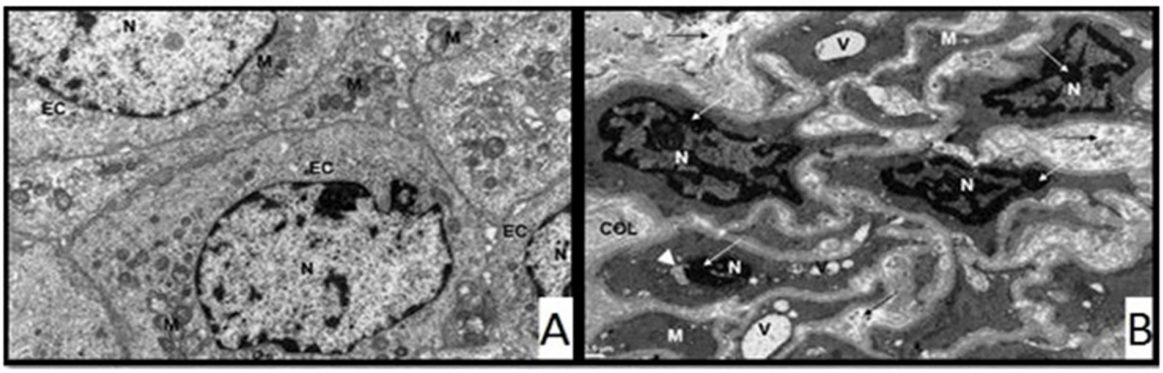

Figure 1A. The sting procedure applied group. Grade 4. Normal transitional epithelial cells (EC) are observed. Nucleus (N), mitochondrion (M). Bar=0,5 µm. **Figure 1B.** The sting procedure applied group. Grade 4. Intercellular edema is observed in the muscular layer (black arrow). Heterochromatin clumping (white arrow) in the nucleus (N) and perinuclear cisternae enlargement (arrow head) in the smooth muscle cells are observed. Swollen mitochondria (M), cristae disorganization in the mitochondria and vacuoles (V) that include membranous whorl structures in the cytoplasm are observed. Collagen (COL). Bar=0,5 µm.

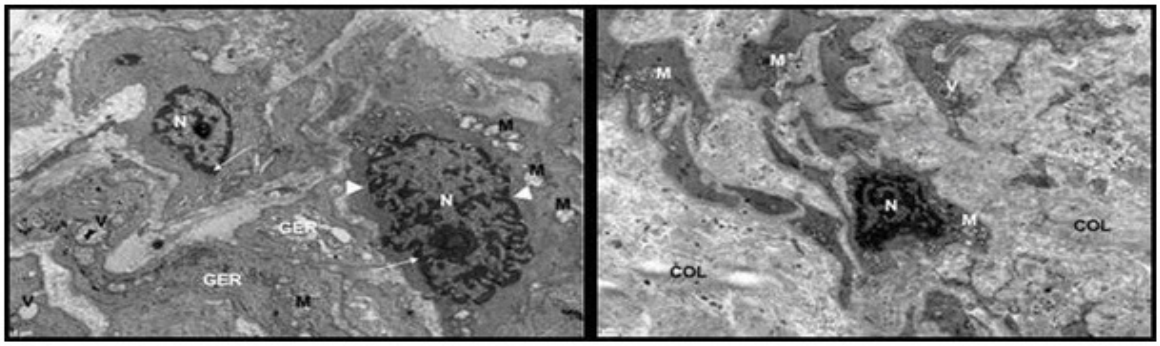

Figure 2A. (Right) The sting procedure applied group. Grade 3. Heterochromatin clumping (arrow) in the nucleus (N) and perinuclear cisternae enlargement (arrow head) in the smooth muscle cells are observed. Swollen granular endoplasmic reticulum cisternae (GER) and mitochondria (M), cristae disorganization in the mitochondria and vacuoles (V) that include membranous whorl structures in the cytoplasm are observed. Bar=0,5 µm. **Figure 2B.** (Left) The sting procedure applied group. Grade 2. Swollen mitochondria (M) and cristae disorganization and vacuoles (V) that include membranous whorl structures in the cytoplasm are observed. Collagen (COL). Bar=1 µm.

pared with the control group ($P < 0.001$, $P < 0.001$). The TGF-β1 levels were significantly higher in patients with VUR compared with the control group (34.2 ± 19.9 vs 5.0 ± 1.9; $P = 0.001$).The amount of VEGF, CD34, and TGF-β1 were not correlated with the grade of reflux ($P = 0.26$, $P = 0.94$, and $P = 0.42$, respectively) (**Table 2**).

DISCUSSION

Several bulking agents have been used for the endoscopic treatment of VUR, and we know that an ideal injectable biomaterial must be easy to inject, nontoxic, and stable without migration to vital organs[8,10-12] .One of these agents, Dx/HA, is formed from cross-linked dextranomer microspheres suspended in a carrier gel of stabilized sodium hyaluronate. The diameters of the microspheres are 80 to 250 µm, and this large size. The histopathological effects of Dx/HA on the intravesical ureters have been evaluated by a few studies, but no electron microscopic study regarding this issue exists in the literature[15-19,24].

The normal ultrastructure of the UVJ in humans was first described by Hanna et al. in 1976[25]. In one rare study by Sofikerim et al. 24 distal intravesical ureteric segments were examined using TEM, reporting normal, similar structures for the tunica mucosa, submucosa and the tunica adventitia in all patients irrespective of the grade of VUR, and pathological findings were observed in only muscular layers[26] . Increasing degree of intercellular edema with increasing grade of VUR and intracytoplasmic vacuoles in grades IV-V were shown semi-quantitatively in the smooth muscle layer and smooth muscle cell structure. It was noted that this degeneration was correlated with the grade of VUR, and the age of the patient had no effect on the results. In our study, using TEM, ultrastructural changes such as intercellular edema in the muscular layer, degeneration and increased cytoplasmic density in the smooth muscle cells were shown in grades II-IV reflux. These 2 studies demonstrate that there is damage to the muscular layer of the UVJ in patients with and without endoscopic injection, and these changes may lead to the dysfunction of cells and their organelles and result in reflux. Consequently, we proposed that this condition might affect the spontaneous resolution of VUR, especially in high-grade patients.

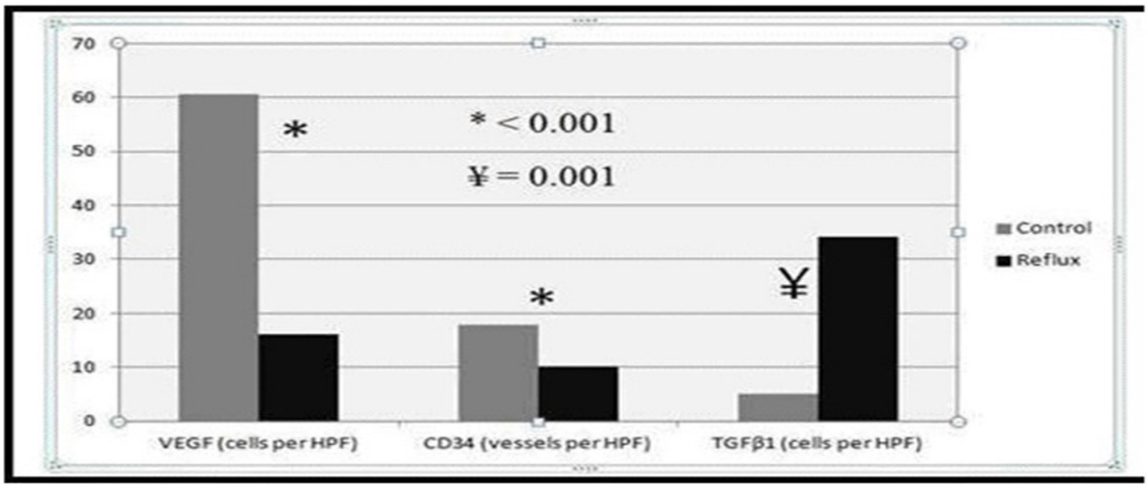

Figure 3. The expression of VEGF, CD34, and TGF-□1 in the control group and patients with reflux. "*" and "¥" indicate statistical significance. Significance was tested using an anova table and a linearity test.

The histopathological effect of Dx/HA injections on distal ureters was first investigated by Stenberg and Lackgren in animal models[19]. Afterwards, the first clinical study was performed by the same authors and included 13 patients with a history of failed endoscopic treatment and 10 patients who had not received Dx/HA; the patients underwent open ureteral implantation and were compared to one another. They concluded that Dx/HA injection is associated with an inflammatory reaction of the giant cell type (100%), chronic periureteral inflammation(33%), and fibrotic pseudo-encapsulation (43%) of the implant[15].

Routh et al. evaluated 16 children who underwent ureteroneocystostomy after failed Dx/HA injection[17]. This was the first study to use the immunohistochemical methods such as CD3, CD20, and MIB-1 staining to examine lymphocyte infiltration and nuclear turnover. They reported slightly increased periureteral inflammation with time and low cell turnover rates (MIB-1), indicating that there was no increase in nuclear proliferation. Ben-Meir et al. investigated the cause of failure of the endoscopic Dx/HA injections[16].Malpositioning of the Dx/HA injections were found in 95% of the examined ureters[16,17] .

In our study, misplacement of the Dx/HA implants was observed in all cases; pseudocapsule formation around the Dx/HA material occurred in 14.3% of the cases in our study, and it was not as frequent as in previously reported studies (43-75%)[15, 17]. We concluded that an abnormal position of the material can explain the failure of endoscopic treatment, but it is difficult to say that this is the exact mechanism. Misplacement of the implant, loss of graft volume by phagocytosis and migration of the injection material were other reported causes of failures[8,16,18,27]. To our knowledge, there is no data showing the histological findings and the localization of the injection material in patients successfully treated with endoscopic procedures.

Schwentner et al. evaluated the extracellular microenvironments and cytokine profiles of UVJs in children with VUR (20). They reported that the amount of TNF-α and TGF-□1 were significantly higher in patients with reflux compared to the control group, while IGF-1, NGF, and VEGF were more abundant in the normal healthy ureters. None of the markers were correlated with age or the VUR grade. In our study, the TGF-β1 levels were significantly higher in patients with VUR compared with the control group ($P = 0.001$).The amounts of VEGF and microvessel density were significantly lower in patients with reflux than the control group ($P < 0.001$,and $P < 0.001$, respectively). The lack of VEGF in the distal intravesical ureters with reflux may be associated with primary VUR because of the smooth muscle disappearance and impaired microperfusion[23, 28, 29]. These results were in parallel to the literature and support the hypothesis regarding the role of ischemia in VUR[20, 21, 26-28]. No significant correlation was observed between the degree of reflux and the amount of VEGF, CD34, and TGF-β1($P = 0.26$, $P = 0.94$, and $P = 0.42$,respectively). We presumed that primary VUR led to this cytokine profile, but it was not possible to show the effect of DxHA injections on the cytokine profile in this study.

In our cases, previous endoscopic injection treatment did not cause significant difficulty during the ureteral re-implantation procedures. This result was similar to published data[10,30]. The bulking agents were typically easily removed en-bloc or in pieces, and care must be taken during the ureteral dissection to avoid ureteral injury. The limitations of this study include the small sample size, the lack of a control group for ultrastructural investigation, evaluation of autonomic innervations and gene mutations as well as limited marker profiles due to restricted financial resources.

CONCLUSIONS

In this study, light and TEM were used to examine the histopathologicaland ultrastructural changes in the ureterovesical junctions of children treated with failed Dx/HA injections for reflux. Subsequently, refluxing ureters exhibits immune-histopathological abnormalities and ultrastructural changes of the muscle cells in all VUR specimens similar to previous reports as menitoned above. It is not easy to determine if these changes were due to the Dx/HA injections or to primary VUR. In further studies, we will examine the distal intravesical ureters of patients with no history of previous endoscopic or open surgery, and we will compare those results with these data.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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