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Comparision of The Histologic Response to Different Bulking Materials Used in Endoscopic Vesicoureteral Reflux Surgery

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Abstract

Objective: To compare the histologic responses of 3 bulking agents, which are used in endoscopic treatment of vesicoureteral reflux (VUR), on rats' urinary bladder and subcutaneous tissue.

Materials and Methods: Thirty rats were divided into 3 groups according to the injection materials; Dextranomer Hyaluronic acid- Deflux®(DxHA-Df), Dextranomer Hyaluronic acid- Dexal®(DxHA-Dx) and Polyacrilate Polialcohol Copolymer-Vantris®(PPC). In each group material was injected both to the submucosa of rats' urinary bladder dome and subcutaneous tissue at their napes. In each group, 5 rats were scarified at 2nd and 6th month of injection. The histopathologic comparement had done by scoring inflammation, neutrophil, eosinophil, macrophage, mast cell and giant cell reaction around the injected material.

Results: All materials were maintained their bulky effect. Despite the big amount of degradation with dextranomer materials, there was minimal degradation with PPC. Materials had the same amount of capsule formation around the injection site, which was not related with the degradation property of the material. There was no statistically significant result for bladder injections. For subcutaneous injections mast cell scores around injection (DxHA-Df, DxHA-Dx, PPC: 1.4, 1.2, 0 respectively. p=0.024) were significantly different at 2nd month. Mast cell scores around injection (DxHA-Df, DxHA-Dx, PPC: 1.0, 1.75, 0 respectively. p=0.007) was significantly different at 6th month also. The inflammation around PPC was higher at 6th month (DxHA-Df, DxHA-Dx, PPC: 1, 1, 3.5 respectively. p<0.05).

Conclusion: Both dextranomer agents were degradable with good capsule formation and minimal inflammation at the adjacent tissue. PPC degraded minimally and caused significant inflammation at adjacent tissue.

Keywords: Vesicoureteral reflux, endoscopic treatment, injection materials

Introduction

In the last two decades, subureteral injection of various bulking agents became the preferred treatment modality of vesicoureteral reflux (VUR). This relatively easy, safe and efficient method increases intravesical length of ureters, narrows the ureter lumen, and fixes the lower end of ureter in bladder wall by local fibrosis. The ideal bulking agent should have a good mound effect at the tissue and preserve this effect for long term without any tissue reaction. Injection should be easy to apply and the material must be stable at its injection area without local or distant migration. The durability of the bulking agent and local tissue reaction are key elements which also has significant impact on the success of treatment.

The success of the endoscopic technique depends on many different factors like the reflux grade (1,2), voiding dysfunction (3), operator (2), and physicochemical properties of the injected material (4). Therefore, clinic studies do not seem suitable in terms of comparing only the injection materials' role on success. The purpose of this animal model is to compare the histological responses of 3 injection materials (2 different dextranomer materials, 1 polyacrylate material) on rat bladder and subcutaneous tissue and interpret the results with possible clinical situations.

Materials and Methods

Animals

The Institutional Care and Use Committee approved the study design, and researchers were accredited by Guidelines of Responsible Use and Human care. A total of 30 healthy male adult Sprague-Dawley rats grown to mean 352 grams (328-424

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gr) and maintained in Hacettepe University Faculty of Medicine Experimental Animal Laboratory in temperature-controlled cages and a dark-light circle with free access to water and food.

Procedure

All injections and surgical interventions were carried out with sterile technique under general anesthesia with intraperitoneal xylazin 5 mg/kg and ketamine 15 mg/kg. Thirty rats were grouped into 3 for each material and sacrificed with carbon monoxide at 2nd and 6th months after the injections.

Three injection materials commercially available were compared in this study:

1.Dexell® (İstem Medikal, Ankara, Turkey (DxHA-Dx): Dextranomer microspheres 50 mg\1 cc, Hyaluronic acid 17 mg\1 cc, Sodium chloride 6.9 mg\1 cc

2.Deflux® (Q-Med AB, Uppsala, Sweden) (DxHA-Df): Dextranomer microspheres 50 mg\1cc, Hyaluronic acid 15 mg\1 cc, Sodium chloride 6.9mg\1cc

3.Vantris® (Promedon, Cordoba, Argentina) (PPC): Polyacrilate polyalcohol copolymer 60%, glycerol 40%.

The same researcher (A.C.B) performed all injections. PPD needles were adapted to commercial injectors containing study materials and scaled by 0.1 cc. Hair on nape and abdomen was shaved. 0.1 cc of materials were injected subcutaneously to midline of napes of the animals. Afterwards, bladders were exposed through 2 cm long vertical suprapubic incisions and emptied with palpation. Bladders were held with atraumatic forceps and traction performed from lateral walls. 0.1 cc of study material was injected to bladder wall at dome. Abdomens were sutured and closed.

Outcomes

The experiments were terminated at 2nd and 6th month. After scarification, injection site in the napes were excised with 0.5 cm lateral margin. Abdomens were re-explored through the previous incision site, and bladders were removed by excising through the bladder neck. Excised tissues were kept in 10% formalin solution and prepared for inspection under light microscope. 5µm thick slides were stained with Hematoxylin-Eosin and Masson's trichrome.

Two histologists who were blinded to the groups examined and photographed the slides. Capsule thickness was measured with Leica Application Suite Programme©. Neutrophils, eosinophils, macrophages, mast cells, and giant cells around the injected material were counted and scored as described by Raut et. al. (5) (Table 1).

Statistical Analysis

Statistical analyses were performed with SPSS 15.0 Programme© and $p<0.05$ was considered as significant. Subgroups (classified according to time of sacrifice) were compared with Kruskal-Wallis tests. Conover's two-sample squared ranks test for equality of variance was used to find out the subgroup leading to the difference.

Results

a. Histological Findings in Bladder Sections

During scarification, in some urinary bladders there were no injection materials at the site of injection. Also, in their histologic sections no injection materials seen. That is most probably the result of thin bladder wall of rats and the leakage of material into the lumen or peritoneum after injection, which was an expected result after our pilot study. That is why we planned also to have injections into the subcutaneous tissue. The missing data was distributed equally into the groups and this error did not effect our statistical comparements. In specimens harboring the materials, biomaterials were concentrated within lamina propria between the epithelial and muscular layers of the urinary bladder, which caused flattening of overlying epithelium and protrusion into the lumen slightly. Microspheres could both seen with full of material or empty at the injection area of each material. Capillaries and cellular infiltration including active fibroblasts and infiltrative cells (especially lymphocytes) and rare multicellular giant cells were observed within and around the microsphere groups (Figure 1). Lymphocytes and occasional mast cells were present outside of the capsule, which was made of collagen fibrils and fibroblasts starting from the 2nd month groups. Degraded empty microspheres could have seen in 2nd and 6th month groups for each biomaterial.

Degradation of DxHA-DX and DxHA-DF were observed to get started earlier and to be completed at 6th month sections. However, degradation of PPC was minimal with persisting full microspheres in 6th month sections.

Table 1. Scoring of inflammatory cells according to Raut et al scoring system (5). (Counted in 1 microscopic field under x40 magnification)

Score	Number of cells counted
0	none
1	1-5 cells
2	6-15 cells
3	16-25 cells
4	≥26 cells

For an objective comparison, infiltration and inflammatory cells were scored, and mean capsule thicknesses were calculated for each group (Table 2 and 3).

Both in 2nd and 6th month sections, all three groups were similar for all parameters (Table 3). Observationally, capsular and intracapsular collagen fibers were thicker, giant cell response was weaker and lymphocytic infiltration around the capsule was prominent in PPC group, however there were no statistically difference among the groups (Table 2 and 3)

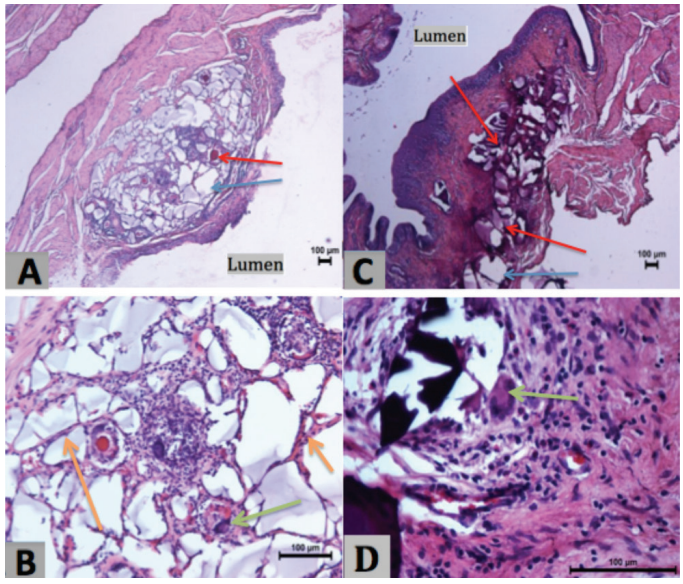

Figure 1. Bladder sections at 6th month. A) DxHA-DF (Deflux) injection site. Most of the microsphers are degraded (blue arrow), unique full microsphers are still exist rarely (red arrow). Bulking effect is seen, flattering of overlying epithelium and protrusion into the lumen slightly. (Hemotoxylin & eosin staining, x25). C) PPC (Vantris) injection site. Most of the microsphers are not degraded (red arrows), unique degraded microsphers are seen (blue arrow). Bulking effect is seen, flattering of overlying epithelium and protrusion into the lumen slightly. (Hemotoxylin & eosin staining, x25). B) Inside of the DxHA-DF (Deflux) injection site. Capillaries and cellular infiltration including active fibroblasts (orange arrows) and infiltrative cells (especially lymphocytes) and rare multicellular giant cells (green arrow) were observed within and around the microsphere groups (Hemotoxylin & eosin staining, x100). D) Inside of the PPC (Vantris) injection site. Capillaries and cellular infiltration including active fibroblasts and infiltrative cells (especially lymphocytes) and rare multicellular giant cells (green arrow) were observed within and around the microsphere groups (Hemotoxylin & eosin staining, x100)

b. Histological findings in subcutaneous tissues

Majority of 2nd and 6th month sections in DxHA-DX and DxHA-DF groups showed complete degradation, whereas degradation was minimal in PPC group (Figure 2).

Although collagen fibers between and around the PPC microspheres seemed thicker, mean capsule thicknesses of three groups were found to be statistically similar (Table 2).

Mast cell infiltration around the capsule was higher in DxHA materials (p=0.024 in 2nd month and p=0.007 in 6th month sections). Overall inflammatory infiltration around the injection site was prominent in 6th month sections of PPC group (p<0.05). Rest of the histologic parameters were statistically the same (Table 3).

Discussion

Majority of the materials used in endoscopic treatment of VUR were abandoned either due to their migration to distant tissues, rapid loss of mass effect, or granuloma formation (4,6). DxHA-Df is a biodegradable material containing cross-linked 80-120µm dextranomer microspheres in stabilized sodium hyaluronic acid carrier medium gel. The gel is absorbed following injections, and microspheres induce a

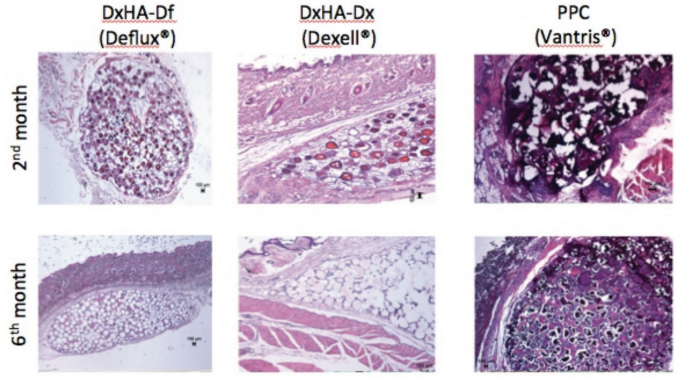

Figure 2. Amount of degradation in subcutaneous tissue of different materials at 2nd and 6th months. For DxHA materials degradation starts early and nearly completes in 6th month. But most of the injection material stays in PPC at 6th month. (Hematoxylin & Eosin, x25 magnification at light microscope, scale at the bottom: 100µm)

Table 2. Median (min-mix) capsule thickness (µm) of the groups. n=number of rats at 2nd/6th months. (Kruskall Wallis Test, p>0.005)

		2nd month	6th month
Urinary bladder	DxHA-DX (n= 2/3)	73.70 (69.20-78.20)	66.77 (64.34-69,20)
	DxHA-DF (n= 3/5)	69.62 (65.48-73.76)	73.22 (68.94-77.33)
	PPC (n= 3/5)	68.59 (66.28-70.90)	73.20 (68.91-75.83)
Subcutaneos tissue	DxHA-DX (n= 5/4)	77.78 (73.60-85.80)	99.23 (95.70-102.47)
	DxHA-DF (n= 5/4)	85.57 (79.59-89.02)	89.22 (86.47-107.81)
	PPC (n= 3/4)	84.11 (80.70-84.52)	114.58 (88.64-131.10)

rapid fibroblast migration and collagen synthesis leading to the capsule formation. While high success rates for short-medium term ranging between 68-92% were reported (4, 7, 8, 9); long-term recurrence rates necessitate to research on new materials. DxHA-DX is another biodegradable dextranomer gel with similar physical and chemical properties. PPC is a non-bio-degradable synthetic material as 320 µm microspheres in glycerol solution. Due to large molecular size, its distant migration is unlikely, and mass effect seems persistent for long term (10).

Microsphere sizes and counts were similar throughout the study period in both tissues and all groups. Degradation of DxHA-DX and DxHA-DF started and were completed earlier than PPC, as expected due to synthetic non-biodegradable nature of the PPC molecule.

Ideally, capsule formation should start early and persist for a long time. Dx-HA materials triggered capsule formation around 2nd month and persisted at the 6th month sections independent from degradation. PPC group, capsule formation and thicknesses were similar to other groups in 2nd and 6th month sections. Also, capsule formations in urinary bladder and subcutaneous tissue sections were similar in all groups (Table 2). Therefore, we can state that quality and persistence of capsule seems unrelated to degradability of the materials and the tissue properties (Figure 3). Ormaecha et. al. reported fibrous capsule thickness reaching 70 µm around non-degraded PPC without significant inflammatory or pathologic infiltration 1 year after injection in dog ureters (10). Researchers attributed low long-term recurrence rate after VUR treatment with PPC to non-biodegradable nature of the material in their clinic study (11). However, we think that such a conclusion can only be possible with prospective randomized trials on many control groups in which other (both biodegradable and non-biodegradable) biomaterials are also used, and clinical success rates or histological features are compared in longer time intervals. Unfortunately, in this study we do not have long-term (1 year or longer) data. Contrary to the findings of Ormaecha et. al. (10), we saw prominent inflammation around PPC in 6th month sections (Table 3).

Although some giant cells could be seen inside the materials, we did not see any granuloma formation both inside and outside of all material injections except one rat's bladder in DxHA-Dx group. As Stenberg A et al. mentions in their study, giant cells are expected within dextranomer injection area with collagen fibers as a result of natural remodeling process (12). Giant cell reaction in the injection site is replaced by fibrosis and connective tissue formation during degradation progress. Mononuclear cell migration, mostly lymphocytes, was infiltrating full microspheres in early sections. As degradation proceeded, this infiltration was replaced by fibroblasts to start collagen formation and forms a honeycomb appearance of empty microspheres. Previously

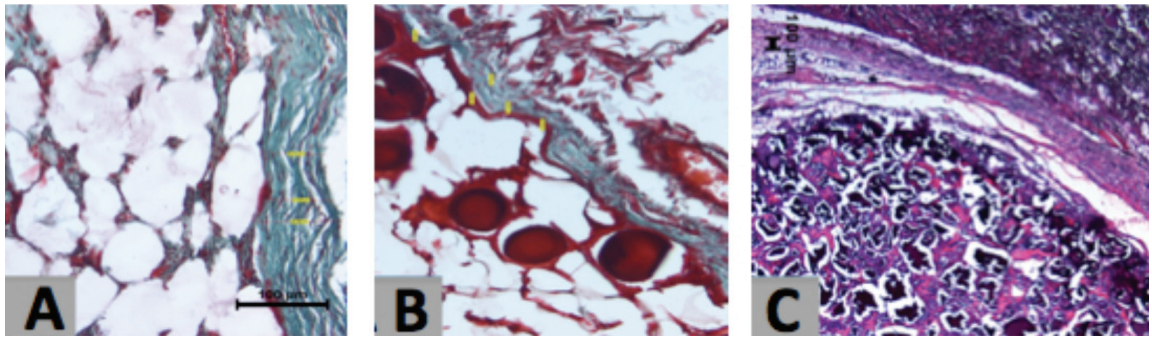

Figure 3. Capsule formations. (6th month, subcutanous tissue). All three materials have statistically equal amount of capsule formation. A. DxHA-Dx (Masson's trichrome x200), B. DxHA-Df (Masson's trichrome x200), C. PPC (Hematoxylene-Eosin x25)

Table 3. Mean scores of histological parameters around enjection materials in urinary bladder and subcutaneous tissue sections (n=Number of biomaterial found rats at urinary bladder/subcutaneus tissue groups), (Kruskall Wallis Test *: p<0.05 and Conover's two-sample squared ranks test to find out the subgroup leading to the difference)

İnfiltration		Urinary Bladder				Subcutaneous Tissue			
		Neutrophils	Eosinophil	Mast cells	İnfiltration	Neutrophils	Eosinophil	Mast cells	
2nd month	DxHA-DX (n=2/5)	0.5(0-1)	0	0.5(0-1)	0.5(0-1)	1.6 (1-3)	0	0.2 (1)	1.2 (1-2)*
	DxHA-DF (n=3/5)	1.3(1-4)	0	1 (0-2)	1(0-2)	3 (3)	0	0.6 (0-1)	1.4 (1-2)*
	PPC (n=3/3)	3.3(3-4)	0.33(0-1)	0	0.33(0-1)	3 (1-4)	0	0	0*
6th month	DxHA-DX (n=3/4)	2 (1-4)	0	0	0.33(0-1)	1 (0-3)*	0	0	1.75 (1-2)*
	DxHA-DF (n=5/4)	1.6(1-2)	0.2(0-1)	0.6(0-1)	1(0-3)	1 (0-3)*	0	0	1 (1)*
	PPC (n=5/4)	2 (1-4)	0	1 (0-2)	0.6 (0-1)	3.5 (3-4)*	0	0	0*

Broderick et. al. attributed failure of endoscopic VUR treatment in a 6-year-old child to phagocytosis of injected material by giant cells shown in histological sections of distal ureters 5 months after the operation (7). Also, Alkan et. al reported granuloma formation in 43.3% of the animals (13). However, we found granuloma formation in only one histological section, which was obtained from urinary bladder of one animal from DxHA-Dx group. Therefore, we think that this pathologic aggravation of giant cell reaction is not a common feature and probably depends on host-specific factors rather than material properties.

Mast cell scores were higher for DxHA groups significantly for both 2nd and 6th months in subcutaneous tissue specimens (Table 3). As known, triptase secretion of these mast cells promotes the conversion of fibroblasts to myofibroblasts which synthesis matrix elements of new connective tissue (14). It was just a slight difference in terms of cell number, which is not pathologic in a normal tissue reaction. Also, eosinophilic infiltration is nearly absent in specimens obtained from all groups in all periods. So, both findings suggest that mast cells are elements of normal inflammatory process rather than an immunologic/allergic reaction, and biomaterials are similar in terms of allergic/immunogenic potential.

Kajbafzadeh et al. compared the short and long-term (1st and 6th months) local tissue reactions against PPC and DxHA-Df in bladders of eight rabbits (15). Inflammation markers (leucocyte common antibody and CD68) were significantly higher in PPC group, both for short and long-term specimens. While mild fibrosis of DxHA-Df group in the short term subsided to non-noticeable levels in the long-term; severe fibrosis of PPC group in the 1st month only decreased to a moderate level in 6th month. Our results overlap this recent study. Also, we establish that a significantly persistent chronic inflammatory reaction continues around PPC material. This finding may point out continuous foreign body reaction and inflammation around non-biodegradable material, which may increase periureteral fibrosis causing an ureteral obstruction.

As we reviewed the literature, common conclusion of authors is to have long term follow up of endoscopically treated reflux patients, to detect reflux failure and also upper urinary tract (UUT) obstructions, for all kind of injection materials. Data about the patients who needed urinary diversions (JJ stents/percutaneous nephrostomies) and open surgical repairs are reported in many endoscopic treatment series (16, 17, 18, 19). Although it is not objective to compare the role of the injection material on obstruction with these published papers, it can be realized that PPC has a higher potential risk of obstruction with less amount of material (20). These histologic responses we detected might be a part of the puzzle for explanation of the relatively higher postoperative obstruction (1.2-6.6%) reported in PPC series (14,21,22). After our rat study, we think that the sustaining inflammation around the PPC injection site can be a possible cause of obstructions. For an objective conclusion we need long-term clinical results of case series, and meta-analysis to compare the results/complications of different materials.

Study Limitation

Small size of animals' urinary bladders led to difficulties in obtaining standard injection volume and exact determination of material volumes. That's why we also injected all materials to the subcutaneous tissue of napes of animals, to reach a nearly same injection volume for an objective outcome. Flattering the overlying bladder epithelium at the injection site is annotated as the maintenance of mass effect.

Like other animal studies, this study also is far from making certain conclusions about the long-term results as maintaining bulking effect, inflammation state, malignancy/immunologic complications. Long-term results of clinical case series, and meta-analysis comparing the results/complications of different materials can help us to have more objective conclusions.

Conclusion

All used biomaterials in the present study formed adequate capsule formation and maintained mass effect throughout the study period without toxic, immunologic, or neoplastic reactions. DxHA was seen to be degraded almost completely whereas PPC was degraded only minimally at the end of the study. However, this finding cannot guarantee long-term effectiveness of PPC and randomized controlled studies conducted in longer periods on larger samples are needed. Long lasting and prominent inflammation around PPC may result in periureteral fibrosis and lack of pliability resulting ureteral obstructions. Histological evaluations of lower ends of ureters in cases that undergo ureteroneocystostomy after failed endoscopic treatments will provide invaluable data.

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