

INJECTABLE SMALL INTESTINAL SUBMUCOSA: PRELIMINARY EVALUATION FOR USE IN ENDOSCOPIC UROLOGICAL SURGERY.

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ABSTRACT

Purpose: We evaluated the possible use of small intestinal submucosa in endoscopic urological surgery by assessing the smooth muscle regenerative capabilities and physical response of various forms of injectable small intestinal submucosa in the canine model.

Materials and Methods: In blinded fashion we injected small intestinal submucosa in 12 dogs submucosally under direct vision using a 20 gauge endoscopic needle. The 4 small intestinal submucosa formulations varied in harvesting method and sterilization technique. Animals were divided into groups of 3 and sacrificed 2 weeks, 6 weeks, 3 months and 6 months after surgery. Each injection site was analyzed grossly and histologically. Smooth muscle regeneration was identified by α -smooth muscle actin immunohistochemical staining.

Results: We identified 2 injectable small intestinal submucosa formulations that induced progressive smooth muscle regeneration at the site of submucosal injection compared with controls. De novo smooth muscle cells appeared in single cell aggregates as early as 6 weeks and in globular aggregates at 3 months. By 6 months early muscle bundle formation was noted. These 2 injectable small intestinal submucosa formulations also had the best submucosal volume preservation of about 25% of injected material during the study period.

Conclusions: Injectable small intestinal submucosa promotes progressive submucosal smooth muscle regeneration in the canine bladder. The combined regenerative and bulking abilities of injectable small intestinal submucosa make this compound unique and novel. The clinical usefulness of injectable small intestinal submucosa for endoscopic correction of reflux and incontinence deserves further investigation.

KEY WORDS: bladder; dogs; mucosa, intestinal; vesico-ureteral reflux; muscle, smooth

During the last 2 decades endoscopic treatment of vesicoureteral reflux and urinary incontinence has been shown to be technically feasible and effective. This technique of submucosal injection of a defined material (polytetrafluoroethylene paste or collagen) may easily be done in an outpatient setting in less than 30 minutes. Unfortunately to our knowledge a universally accepted injectable material has not yet been identified, which has limited widespread clinical use. Ideally the perfect substance would be readily obtained, easily injected, nonimmunogenic, nonmigratory and efficacious in the long term. Another attractive characteristic of an injectable substance would be the induction of tissue regeneration of functional muscle by the injected material as well as collagen deposition in an area of deficiency. Small intestinal submucosa may be such a substance.

Small intestinal submucosa is a naturally occurring, acellular extracellular matrix derived from porcine small intestine. In the intact form this unique material retains its 3-dimensional architecture and contains collagen, proteoglycans, glycosaminoglycans and functional growth factors, which have been shown to promote tissue growth and differentiation.¹ In various tissue engineering applications small intestinal submucosa has induced organ specific tissue regeneration.^{2,3} In urological applications this substance has induced xenographic regeneration of the bladder in vivo when used for augmentation in animal models.⁴ Regenerated bladder was almost indistinguishable from normal bladder

histologically and physiologically.⁵ We investigated whether an injectable form of small intestinal submucosa would also induce bladder regeneration at the site of injection and, therefore, be a potentially useful substance in endoscopic urological surgery.

MATERIALS AND METHODS

A total of 12 dogs were used in our study according to our institutional animal care protocol. Each dog received general halothane anesthesia and submucosal injection of small intestinal submucosa under direct vision via anterior cystotomy. A dose of 0.5 cc per bleb of 4 blinded porcine small intestinal submucosa formulations was injected submucosally in blinded fashion using a 20 gauge endoscopic needle into the bladder of each animal and marked geometrically on the serosal surface by a permanent suture for future identification. An injection of 100% glycerin served as a control injection in each case. The injection was made supratrigonally and in the body of the bladder in each animal. Each small intestinal submucosa formulation was suspended in a glycerin-phosphate buffer carrier and labeled only by lot number. At the time of injection ease of injection and entry site leakage were noted for each formulation. The specific processes of manufacturing these formulations are proprietary to the manufacturer but all paste formulations were mechanically processed in a similar manner. Formulations differed with respect to the age of the small intestinal submucosa source (sow versus butcher age) and the sterilization method (aseptic processing with or without E-beam irradiation). Investigators were blinded to the formulation used at

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injection. After injection bladders were closed in 3 layers. Catheter drainage was not used postoperatively.

Animals were divided into groups of 3 and sacrificed at 2 weeks, 6 weeks, 3 months and 6 months after surgery. Bladders were harvested en bloc with the remainder of the surrounding tissue and then opened through the previous anterior cystotomy. Submucosal bleb size was grossly assessed before dissecting the injection area. After fixation in buffered formalin we performed routine histological hematoxylin and eosin analysis. Masson trichrome stained sections were also used to identify residual and/or deposited collagen, and assess the location of the injected material. To assist in identifying the areas of smooth muscle regeneration at injection sites we performed immunohistochemical staining using a monoclonal antibody to α -smooth muscle. All sections were compared with those of the glycerin injections and normal bladder sections at the respective time points. Histological observations were graded using a consistent system (see table). Smooth muscle infiltration of the injection site was classified as 0%, less than 10%, 10% to 25%, 25% to 50% and greater than 50%. Volume preservation grade was catego-

rized as a percent estimate of the original 0.5 cc injected volume as 10% or less, 25%, 50% to 75% and 75% or greater.

RESULTS

Technically all emulsified small intestinal submucosa preparations were easily injected through a 20 gauge endoscopic needle with minimal to no leakage. A well formed bleb of injected small intestinal submucosa was consistently created after 0.5 cc was injected. There were no intraoperative or postoperative complications and all animals survived to the respective time points. At sacrifice the bladders were easily removed en bloc with a minimal postoperative reaction. Injection sites were easily identified grossly and/or by marking sutures.

At 2 weeks all injection sites were marked by a moderate inflammatory and edematous reaction within the injection matrix (fig. 1, *A* and *B*). However, during the study period the injected matrix became a more discrete and concentrated histological nodule, and was easily identified by the submucosal collagen mass visualized on Masson trichrome stained

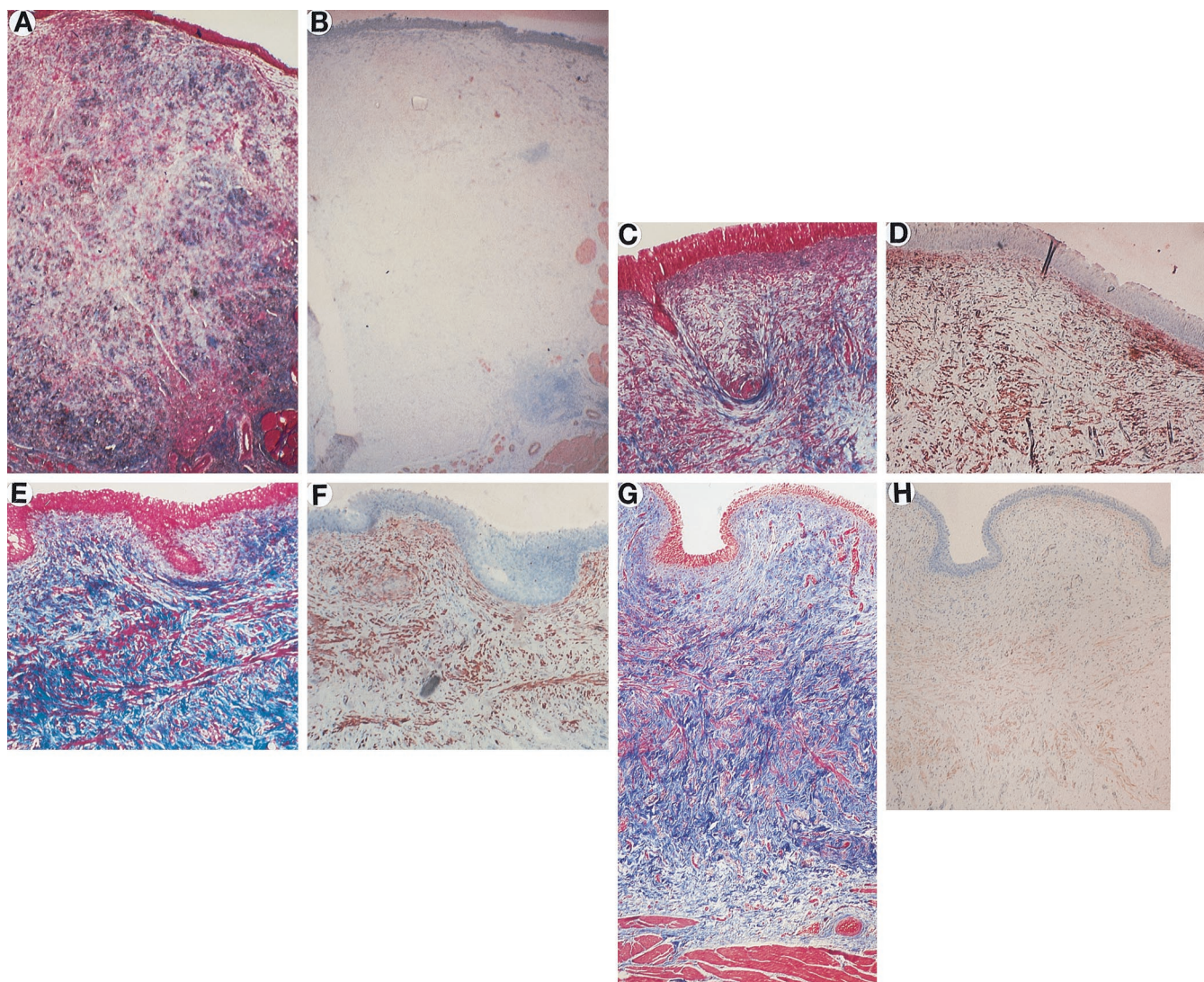


FIG. 1. Canine bladder submucosal histology after small intestinal submucosa injection. *A* and *B*, at 2 weeks inflammatory and edematous reaction developed within large submucosal bleb of injected matrix. Reduced from $\times 40$. *C* and *D*, at 6 weeks de novo infiltrating smooth muscle cells are loosely arranged and distinct from muscularis mucosa. Reduced from $\times 40$. *E* and *F*, at 3 months de novo smooth muscle cell density is increased and cells are arranged in globular aggregates within defined submucosal collagen nodule. Reduced from $\times 40$. *G* and *H*, lower magnification shows full thickness of submucosal infiltration of de novo smooth muscle cells at 6 months with further increased cell density and cells apparently organized into early bundle formation throughout preserved submucosal nodule. Reduced from $\times 20$. *A*, *C*, *E* and *G*, Masson trichrome stain. *B*, *D*, *F* and *H*, α -smooth muscle immunostained sections with red cells demonstrate α -smooth muscle actin positivity.

sections (fig. 1, *E* and *G*). As early as 6 weeks, de novo spindle shaped cells started to infiltrate the injected mass. Spindle cells began to appear as loosely arranged, horizontally positioned single cell aggregates. These spindle cells were located at the submucosal apex and were distinct from the muscularis mucosa (fig. 1, *C*). They were not observed in the lamina propria in this degree in normal sections. Infiltrating spindle shaped cells accumulated with time in a progressive manner. At 3 months the spindle shaped cells were arranged in globular aggregates within the injected mass collagen network (fig. 1, *E*). At 6 months they appeared to be organized into early bundle formation and were diffused throughout the submucosal nodule (fig. 1, *G*).

Two formulations consistently induced superior spindle cell formation and infiltration compared with the other 2 (see table). After unblinding to the processing of the 4 formulations we noted that the 2 inducing the greatest amount of spindle shaped cell infiltration had been sterilized by E-beam irradiation. Age of the animal from which the small intestinal submucosa was derived (sow versus butcher age) had no influence on the degree of spindle shaped cell infiltration. Notably glycerin control sites had minimal to no spindle shaped cell formation, which never approached that induced by small intestinal submucosa.

All spindle shaped cell infiltrates noted on hematoxylin and eosin, and Masson trichrome sections stained positive for α -smooth muscle actin (fig. 1). This finding supports the hypothesis that these spindle shaped cells had a smooth muscle phenotype. Normal sections had few α -smooth muscle actin positive cell in the lamina propria (fig. 2). Glycerin controls also had minimal to no α -smooth muscle actin positive cells in the lamina compared with those induced by small intestinal submucosa. At 3 and 6 months glycerin control sites were indistinguishable from normal noninjected sites.

Compared with results in normal sections glycerin appeared to cause a more severe, early inflammatory and edematous reaction than any small intestinal submucosa formulation. However, there was a mild inflammatory infiltrate within the lamina propria with occasional lymphoid formation at all injection sites. Inflammation and edema at all sites began to resolve at 6 weeks and had disappeared by 3 months. Neovascularity was evident as early as 2 weeks within the injection nodule of small intestinal submucosa. This neovascularity did not progress and was never prominent at any later point. All injection sites were identified by scar and/or the submucosal collagen mass at all time points. We did not determine whether this collagen was newly deposited or residual from the initial small intestinal submucosa injection.

Most injections retained a gross nodular appearance at 2 weeks with 75% to 50% volume preservation (see table and

fig. 3, *A*). This finding was in contrast to the glycerin blebs, which had uniformly disappeared grossly by this time. During the ensuing 6 months the 2 formulations that had been subjected to E-beam irradiation and histologically had the best induction of smooth muscle also retained the most volume grossly (fig. 3, *B*). This result was shown by distinct submucosal thickening of approximately 25% of the initial injection volume. The remaining formulations had a greater degree of volume loss during the study and were not easily identified grossly at 6 months.

DISCUSSION

Open surgical correction of vesicoureteral reflux and urinary incontinence has been the treatment of choice when clinical improvement is not achieved by medical therapy. However, during the last 2 decades endoscopic correction of these conditions has challenged traditional open methods. The majority of clinical experience with endoscopic correction of reflux and incontinence has been with polytetrafluoroethylene paste. Periureteral and periurethral injections of polytetrafluoroethylene as a bulking agent are technically feasible and efficacious.⁶⁻¹² Long-term resolution rates of reflux and incontinence with polytetrafluoroethylene approach 85% and 60%, respectively. Unfortunately polytetrafluoroethylene has been shown to undergo distant particle migration with subsequent granulomatous reaction, calling into question the safety of this material.¹³⁻¹⁶ Other inorganic substances and systems have also been studied, such as silicone micro-implants, polyvinyl alcohol, glass particles, dextranomer microspheres in sodium hyaluronan solution and detachable balloon systems.¹⁷⁻²¹ Again, to date none of these substrates has been biocompatible (safe, nonimmunogenic and nonmigratory) and efficacious in the long term.²² As an alternative to inorganic substrates, glutaraldehyde cross-linked bovine collagen also has been used. Short-term resolution rates of reflux and incontinence approach those of polytetrafluoroethylene.^{23,24} However, in the long term efficacy is decreased, which is likely secondary to subsequent degradation and volume loss of the injected material with time. In an effort to overcome the problems of particle migration and degradation others have used autologous materials for injection. Injected substances such as autologous collagen, fat, smooth muscle cells and chondrocyte alginate suspension were effective at early followup but long-term efficacy remains to be demonstrated.²⁵ Chondrocyte alginate suspension is attractive as a bulking agent and demonstrates the usefulness of transplanted cells in this setting. However, this technique requires cartilage biopsy and chondrocyte cell culture expansion, which may be costly and not readily available to all clinicians. Injectable small intestinal submucosa

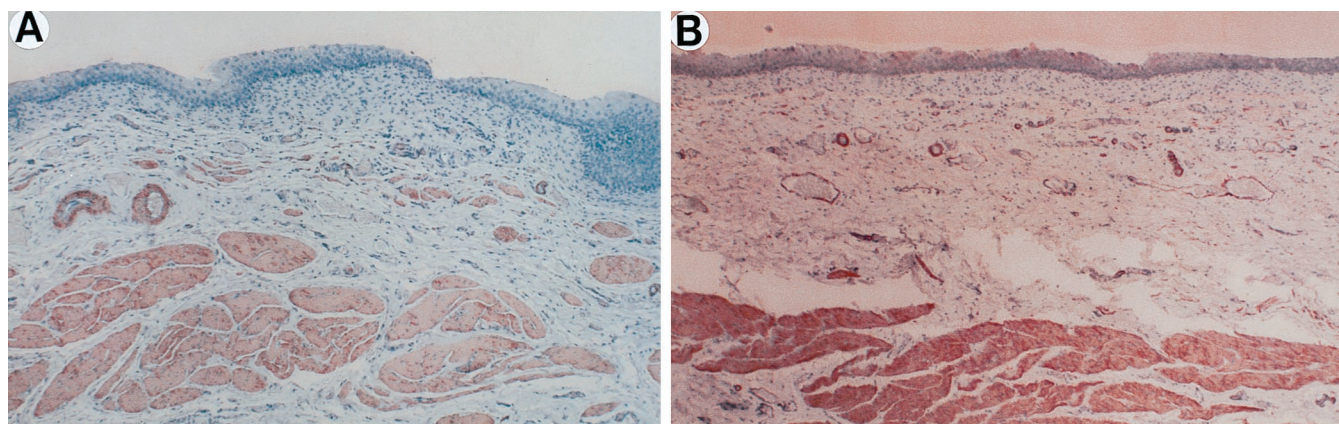


FIG. 2. *A* and *B*, normal canine bladder histological sections show submucosal bladder architecture. Note absent smooth muscle cells in lamina propria. Red areas indicate smooth muscle. α -Smooth muscle immunostaining, reduced from $\times 20$.

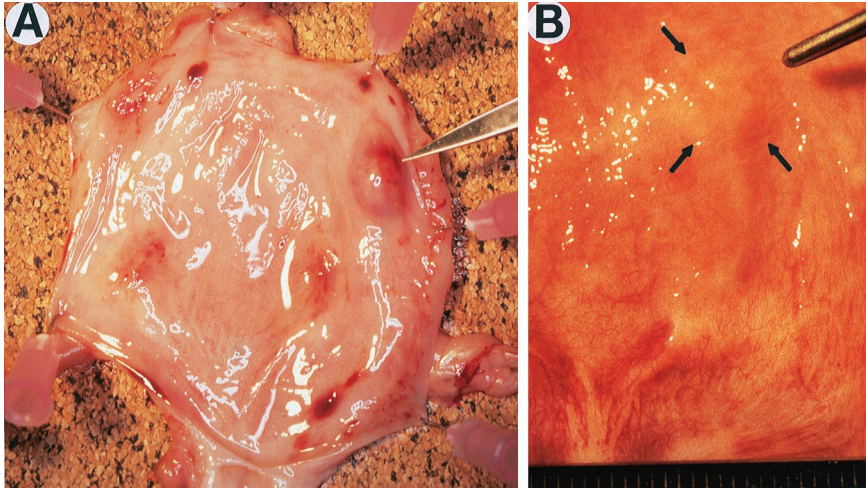


FIG. 3. Canine bladder injection sites of small intestinal submucosa since time 0 at sacrifice. A, at 2 weeks spherical nodule of injected small intestinal submucosa represents greater than 75% volume preservation. B, at 6 months small but distinct tissue nodule (arrows) represents approximately 25% volume preservation.

appears to be advantageous in that it involves autologous cell regeneration, is readily available and does not require processing for each patient. Histological observations were graded by a consistent grading system without the need for in vitro cell culture techniques.

Knapp et al initially assessed an injectable form of small intestinal submucosa using canine small intestinal submucosa in a porcine bladder model.²⁶ They documented that a submucosal injection of small intestinal submucosa produced neoconnective tissue composed of spindle shaped cells at the injection site. In a canine reflux model Safir et al injected a porcine form of small intestinal submucosa.²⁷ Reflux was corrected after injection in 5 of 6 dogs. However, reflux had recurred to some degree in all animals at sacrifice. Important endoscopic observations made in this pilot study were that the previous small intestinal submucosa formulation used did not form a well visualized bleb at the injection site, the formulation leaked out of the needle tract after injection and surgically created reflux in the canine model is unlike the natural condition in humans. The former 2 observations are likely associated with the viscosity of the small intestinal submucosa suspension. However, histological examination revealed that injectable small intestinal submucosa induced collagen deposition and the ingrowth of new onset spindle cells that were positive for α -smooth muscle actin. This find-

ing was the initial indication that small intestinal submucosa may not only act as a bulking agent like other injectable substances, but also promote the regeneration of native tissue at the injection site.

The aforementioned initial studies of injectable small intestinal submucosa prompted our series, in which we evaluated 4 formulations of small intestinal submucosa with superior paste-like characteristics. To eliminate the possible experimental variables of an animal reflux model we placed injectable small intestinal submucosa submucosally in normal canine bladders, which enabled us to focus on the ability of the formulations to induce smooth muscle regeneration and submucosal collagen deposition. In our study the tissue regenerative capacity of injectable small intestinal submucosa was demonstrated by the induction of new onset smooth muscle cells within the matrix of the injection site. This regeneration of smooth muscle cells was progressive and developed without the need for previous seeding with autologous cultured cells. To our knowledge tissue regeneration as a result of an injected matrix is a unique and novel property not noted with any other injectable substance studied to date. The application of such a tissue engineering technique may most likely not be limited to urology.

In our study we also evaluated small intestinal submucosa grossly and histologically as a bulking agent. Two formulations retained about 25% of injected submucosal volume for up to 6 months (fig. 3, B). The submucosal nodule consisted of collagen and new smooth muscle formation. We did not evaluate whether the submucosal collagen was newly deposited or residual from the initial injection. However, it likely represented a combination of each type, since in previous studies of intact small intestinal submucosa there was new collagen in the regenerated tissue. Some degree of volume loss is not surprising since the major components of small intestinal submucosa are extracellular matrix proteins that are broken down and reabsorbed with time. In addition, Badylak et al reported that the nonirradiated type of intact small intestinal submucosa is completely reabsorbed by as early as 4 to 6 weeks.²⁸ In our study the 2 injectable formulations with the best volume preservation and smooth muscle induction were irradiated, which implies that E-beam irradiation may prolong the smooth muscle induction and reabsorptive process of this matrix. Radiation at a molecular level causes protein denaturing and nucleic acid fragmentation in sterilization doses. Why E-beam irradiation provides better tissue regeneration and volume preservation may only be speculated upon at this time. It may be secondary to partial collagen

Histological grading system of smooth muscle induction and bulking capacity of injectable small intestinal submucosa in the canine bladder

Formulation	Sacrifice	% Smooth Muscle Induction	% Bulking Capacity
1 E-beam	2 Wks.	0	50–75
	6 Wks.	Less than 10	25
	3 Mos.	10–25	25
	6 Mos.	Greater than 50	25
2 E-beam	2 Wks.	0	50–75
	6 Wks.	Less than 10	25
	3 Mos.	10–25	25
	6 Mos.	25–50	25
3 No E-beam	2 Wks.	0	75 or Greater
	6 Wks.	0	10 or Less
	3 Mos.	Less than 10	10 or Less
	6 Mos.	Less than 10	10 or Less
4 No E-beam	2 Wks.	0	75 or Greater
	6 Wks.	Less than 10	10 or Less
	3 Mos.	0	10 or Less
	6 Mos.	Less than 10	10 or Less

cross-linking, which enables the matrix to resist absorption. This possibility and others require further investigation.

The mechanism by which injectable substances correct reflux and/or incontinence theoretically depends on the physical material as a bulking agent to change the anatomy of the ureterovesical junction or bladder neck. Injectable small intestinal submucosa may be beneficial in other ways in addition to its usefulness as a bulking agent. Due to the induction of smooth muscle regeneration by small intestinal submucosa the additional potential benefit of functional muscle at a site of anatomical deficiency cannot be overemphasized. This new muscle formation may be functionally more beneficial than the bulking properties of small intestinal mucosa. It is tempting to speculate that the development of this autologous smooth muscle at the injection site may be permanent. The exact functional role of this new muscle has not yet been defined and is currently being evaluated in a porcine reflux model. In addition, future studies to evaluate the exact mechanism of tissue specific regeneration must be performed.

Small intestinal submucosa has been shown to be biocompatible and nonimmunogenic as a xenograft in many nonurological applications in humans, including in the aorta, vena cava, skin, cartilage, tendon, bone and skeletal muscle, and it is a material approved by the Food and Drug Administration.²⁹ Recently porcine small intestinal submucosa was used in humans as a graft for anterior cruciate ligament regeneration in reconstructive knee surgery. In our canine bladder model histological examination revealed that small intestinal submucosa evoked a minimal immunological response, as in previous studies.^{26,27} The need to evaluate this substance for possible compositional migration has not been an issue since small intestinal submucosa is composed of naturally occurring proteins, all of which are biodegradable. To date none has been shown to cause any significant immunological or any granulomatous reaction.

CONCLUSIONS

Injectable small intestinal submucosa promotes submucosal smooth muscle regeneration at the injection site in canine bladders. This new onset smooth muscle formation increased progressively during the 6-month study period. The combined regenerative and bulking abilities of injectable small intestinal submucosa make it a unique and novel compound. The clinical usefulness of injectable small intestinal submucosa for the endoscopic correction of reflux and incontinence deserves further investigation.

Porcine small intestinal submucosa formulations were provided by Cook Biotech, Inc., West Lafayette, Indiana.

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EDITORIAL COMMENT

The authors report on the use of an injectable form of small intestinal submucosa in a canine bladder model. Like many acellular extracellular matrix substances, small intestinal submucosa has received a great deal of attention in recent years in urology. Other potential applications reported include bladder augmentation, hypospadias repair and bladder neck suspension (reference 12 in article).¹ The focus of this report was the evaluation of 4 preparations of injectable small intestinal submucosa in regard to bulking capacity, durability and ability to induce smooth muscle tissue. Injectable

small intestinal submucosa formulations in this study differed with respect to the sterilization technique and time of harvest of the porcine tissue. The 2 irradiated preparations resulted in better bulking capacity durability as well as increased smooth muscle induction than the nonirradiated formulations. Porcine small intestinal submucosa age did not have any impact.

Less surgical invasive techniques for treating vesicoureteral reflux and urinary incontinence have been popular during the last 2 decades. Unfortunately major limitations of injectable substances include the immunogenic impact as well as the durability and success of these substances. As the authors noted, granulomatous formation and migration have been reported (references 13 and 14 in article). Furthermore, long-term success has not equaled that of surgical therapy. Similar to previous substances, small intestinal submucosa bulking capacity appears to decrease with time, as demonstrated in the initial 6 weeks of this study. Interestingly this decrease was less with irradiated small

intestinal submucosa at further followup and it seemed to plateau and remain stable. Furthermore, smooth muscle induction was more prominent in these irradiated formulations with longer followup. It would be interesting to follow these animals even further.

Although this prospective, single blinded, controlled study shows great potential for treating vesicoureteral reflux and urinary incontinence, I agree with the authors that the clinical usefulness of injectable small intestinal submucosa deserves further investigation. Injectable small intestinal submucosa and similar compounds undoubtedly will have an exciting role in the development of future urological reconstruction.

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