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Development of a rabbit's urethral sphincter deficiency animal model for anatomical-functional evaluation

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ABSTRACT

Objective: The aim of the study was to develop a new durable animal model (using rabbits) for anatomical-functional evaluation of urethral sphincter deficiency.
Materials and Methods: A total of 40 New Zealand male rabbits, weighting 2.500 kg to 3.100 kg, were evaluated to develop an incontinent animal model. Thirty-two animals underwent urethrolysis and 8 animals received sham operation. Before and at 2, 4, 8 and 12 weeks after urethrolysis or sham operation, it was performed cystometry and leak point pressure (LPP) evaluation with different bladder distension volumes (10, 20, 30 mL). In each time point, 10 animals (8 from the study group and 2 from the sham group) were sacrificed to harvest the bladder and urethra. The samples were evaluated by H&E and Masson's Trichrome to determine urethral morphology and collagen/smooth muscle density.
Results: Twelve weeks after urethrolysis, it was observed a significant decrease in LPP regardless the bladder volume (from 33.7 ± 6.6 to 12.8 ± 2.2 cmH_2O). The histological analysis evidenced a decrease of 22% in smooth muscle density with a proportional increase in the collagen, vessels and elastin density ($p < 0.01$).
Conclusions: Transabdominal urethrolysis develops urethral sphincter insufficiency in rabbits, with significant decrease in LPP associated with decrease of smooth muscle fibers and increase of collagen density. This animal model can be used to test autologous cell therapy for stress urinary incontinence treatment.

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INTRODUCTION

Stress urinary incontinence (SUI) is a high prevalent condition with great economic and quality of life impact (1). Loss of adequate anatomic urethral support and intrinsic sphincter deficiency (ISD) are the two major components related to the development of stress urinary incontinence (SUI). Surgical techniques are effective to repair the anatomic support defect. However, the treatment of ISD component is a much more complex issue. The intrinsic sphincter accounts for approximately 50% of the continence mechanism and incontinence occurs as a consequence of decreased activity of the smooth muscle of the intrinsic urethra.

In the past, different approaches have been described to improve the urethral continence. One minimally invasive alternative was the injection of bulking agents (2). Several materials were tested with different success rates (3,4). However, none of them sustained significant results according to time (5). The lack of an ideal substance to use as bulking agent and the potential to urethral function regeneration by means of cell therapy and

tissue engineering lead to studies based on cells transplantation into the urethral wall and rhabdosphincter (6-8). However, such therapies are associated with major potential risks that should be extensively evaluated before clinical application.

One of the biggest challenges to develop new SUI treatments is the lack of an ideal animal model to test such treatments. This is particularly true when planning to develop a successful cell transplantation therapy for a non life-threating disease. An animal model to test cell injections should allow: reproducible injection in an adequate thick urethral wall, an easy and reproducible evaluation of functional and morphological regeneration/recuperation, an autologous transplantation, a mid and long term follow-up and the evaluation of possible cell migration teratogenicity and mutagenicity.

The majority of SUI animal models were developed in rats. However, low bladder capacity, presence of early detrusor contraction, small urethral wall thickness and small diameter make the cells injections and functional analysis of such animals very complicated with several possible biases. Furthermore, the urethral cell injections described in the literature (9) were performed either with isogenic or immunossupressed animals and it is much more feasible to perform autologous cell transplantation in rabbits than in rats. On the other hand, rabbits are bigger animals, with larger bladder capacity, shorter relative functional urethra, thicker urethra wall, larger urethral lumen, stable bladder and allow autologous cells transplantation. In the present study, we examined the possibility to develop a urethral sphincter deficiency animal model in rabbits that would be suitable for the study of urethral cell injections.

MATERIALS AND METHODS

The present study was conducted after approval and following all requirements from the Ethical Committee of our institutions.

Developing urinary incontinence model

After the initial evaluation, a total of 40 New Zealand male rabbits, weighting 2.500 kg to 3.100 kg were used to develop an incontinent animal model to test cell therapy.

The study was designed to determine if a standardized peri-urethral lesion (urethrolysis) would determine urethral functional and morphological changes, as previously described in rats (10). Thirty two animals underwent urethrolysis and 8 rabbits were kept as a sham group. Animals underwent cystometry and LPP determination, before surgical procedure and at 2, 4, 8 and 12 weeks after intervention. In each of the previous mentioned time point, 10 animals (8 animals from the study group and 2 animals from the sham group) were sacrificed to harvest the bladder and urethra.

Anesthesia, analgesics and antibiotic prophylaxis

For the cystometric and LPP evaluation, the rabbits were anesthetized using 20 mg/kg of intramuscular (IM) Ketamine, 10 minutes prior the study. Transabdominal urethrolysis was performed after the first cystometric evaluation, using 4 mg/kg of additional intramuscular Xylazine. Whenever necessary, we used a maintenance dose of 5 mg/kg of Ketamine and 1 mg/kg of Xylazine (IM) during the surgical procedure.

Antimicrobial prophylaxis was carried out with 100 mg of sodium Cefalotine (IM), in a single dose, one hour before the surgery. Postoperative analgesia was carried out with 100 mg of sodium Dipirona (IM), every 8 hours, for 2 days.

To harvest bladder and urethra, the animals were anesthetized similarly to the urethrolysis, being sacrificed by an intravenous high anesthetic dose.

Cystometric evaluation and LPP determination

A 4 Fr. catheter was placed inside the bladder through the urethra. The bladder was emptied and the catheter was connected to a three-way stopcock that allowed connection to the infusion pump (Harvard Apparatus model P-22) and to the pressure transducer (Viotti 5600/ Urosystem DS-5600 version 4.52- Viotti & Assoc. - Sao Paulo - Brazil). The bladder was filled out at a continuous rate of 3 ml per minute (about 10% of the blad-

der capacity), with methylene blue diluted in saline (1:1000) to facilitate determination of urinary leakage (10). The bladder capacity was determined by filling it out until bladder contraction and micturition ocurred.

During the filling phase, abdominal pressure was slowly increased by gentle and continuous pressure with 2 fingers placed on the lower abdomen directly over the bladder until one drop was observed through urethral meatus (10). As soon as the leak was observed, the pressure was withdrawed and the bladder pressure rapidly returned to baseline, demonstrating that leakage was not associated to bladder contraction.

The pressure in the bladder at the exact moment that a drop was visualized on the urethral meatus was considered as LPP. This measure was performed three times (the average value was used to statistical analysis) in three different moments of the filling phase; after infusion of 10, 20, and 30 mL.

Transabdominal urethrolysis

The urethrolysis was performed as previous described in rats (10). Briefly, after anesthesia and adequate antiseptic preparation, a suprapubic midline incision of 4 cm was performed in order to expose the bladder urethra. In the sham group, the incision was closed after complete exposure of the bladder and urethra. To perform the urethrolysis, we identified the ureter bilaterally and carried out the dissection of the entire urethra. Careful was taken to avoid vesical arteries and ureteral injury. To totally release the urethra, we opened the endopelvic fascia and sectioned the pubic-urethral ligaments on both sides, dissecting the urethra circumferentially from its proximal to its distal point. The incision was closed in 2 separated layers using Vycril 4.0 in the abdominal wall and nylon 4.0 in the skin.

Histological analysis

After 2, 4, 8 and 12 weeks, the animals were sacrificed, the urethra and bladder were formalin fixed, paraffin embedded and cross-sectioned in 5 µm sections. The slides were stained with Hematoxilin-Eosin (H&E) and Masson's Trichrome. Stereologic evaluation was performed to evaluate collagen and smooth muscle fibers density. Stereology is a method that uses systematic and randomized samples, counting the collagen and smooth muscle fibers density. The collagen and smooth muscle fibers were analyzed and counted in 100 random sterology microscopical fields under great magnification (400 X), of the urethra cross-section for each animal using Olympus microscope BX-51, with differential interference contrast. Only one person analyzed the histological slides.

Statistics evaluation

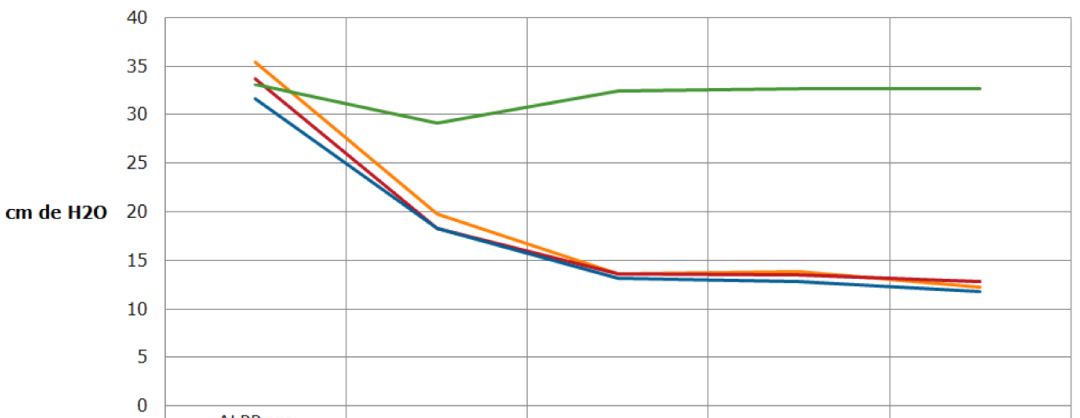
The results were analyzed considering the type, distribution and nature of the variables using parametric or nonparametric tests. It was applied the Wilcoxon's or Friedman's analysis of variance to compare pre and postoperatively LPP values in different timepoints for each animal. The stereological data were evaluated by Kruskal-Wallis' test. Statistical significance was determined at p values < 0.05.

RESULTS

The average functional bladder capacity pre and 12 weeks post urethrolysis was 34.5 ± 6.4 mL and 36.2 ± 7.4 mL, respectively ($p = 0.185$). After urethrolysis, there was a progressive decrease in the ALPP. It becomes more evident with significant decrease ($p < 0.05$) at 2 weeks after urethrolysis (Figure-1). The pre and post urethrolysis LPP evaluation with different bladder distension and at different time points are shown in Figure-1. The bladder volume did not interfere in the LPP value as shown in Figure-1. There was no significant difference between the control groups at various stages of study (Figure-1).

At 4 weeks, the LPP was similar to the LPP at 8 and 12 weeks ($p > 0.05$ - Figure-1). In the same way, there was a progressive increase in collagen and decrease in smooth muscle in the urethra wall. The control group presented unchanged collagen/smooth muscle distribution in the urethra wall. The animals submitted to ure-

Figure 1 – Average ALPP after filling the bladder with 10, 20 and 30 mL at different timepoints: pre-urethrolysis 2,4,8 and 12 weeks after urethrolysis. Sham group with 20 ml bladder distension



	ALPP pre urethrolysis	ALPP 2 weeks	ALPP 4 weeks	ALPP 8 weeks	ALPP 12 weeks
10 ml	35.4	19.8*	13.6*	13.8*	12.3*
20 ml	33.7	18.3*	13.6*	13.5*	12.8*
30 ml	31.7	18.3*	13.2*	12.8*	11.8*
Sham Group	33.1	29.1	32.5	32.7	32.7

*p<0,05 – Compared with Sham group.

throlysis developed an increase in collagen and decrease in smooth muscle distribution that became evident after 2 weeks (Figure-2 - $p < 0.05$). Figure-3 shows a Masson´s Tricrome staining at the different time points.

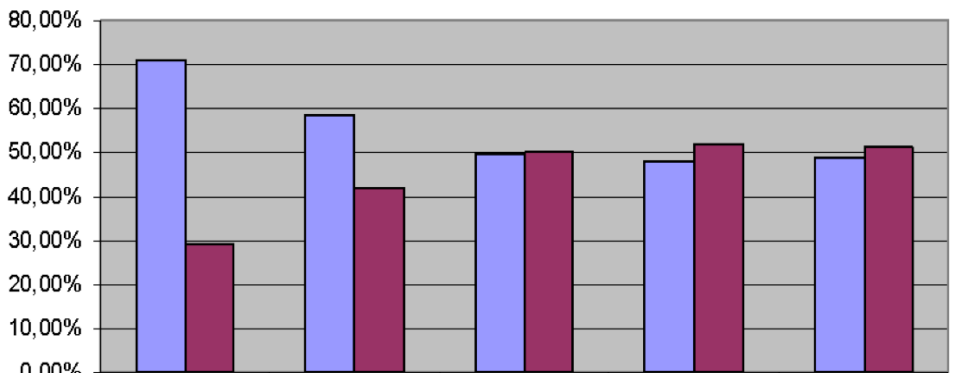
DISCUSSION

The main purpose of the present study was to develop a durable and easily reproducible animal model that would allow testing autologous cellular therapy injection in the urethra wall. Rabbits allow autologous cell harvesting, have good endurance on surgery, a thick urethra wall, stable bladder and a good bladder capacity. Thus, we developed a functional and anatomical urethral sphincter lesion in male rabbits. This animal model was demonstrated to be durable and easily reproducible. By performing urethrolysis, we created a functional damage demonstrated by a progressive and sustainable decrease in the LPP that was associated with decrease in urethral musculature density.

Several methods, which mimic a variety of clinical urodynamic tests, have been developed to determine urethral resistance in animal models of SUI. The majority of animal models were developed in rats. One of the most used methods to evaluate urethral resistance in rats is the determination of LPP (11). In these animal models, the intravesical pressure can be evaluated by urethral or suprapubic catheter (12,13). However, to determine the LPP in rats requires a very well trained investigator to eliminate confounding variables, especially because the LPP evaluation can trigger micturition and urethral catheter may increase the urethral resistance (14).

In 1998, an animal model for SUI was developed in the attempt to simulate one of the known risk factors for the development of SUI. The authors used an intra-vaginal balloon to simulate birth vaginal trauma with or without ooforectomy (15,16). However, it was not effective in create a reproducible method. LPP decreases to approximately 60% of control values 4 days after simulated birth injury (17). When incontinence was

Figure 2 - Muscle and collagen distribuition (%) in the wall at different time points.

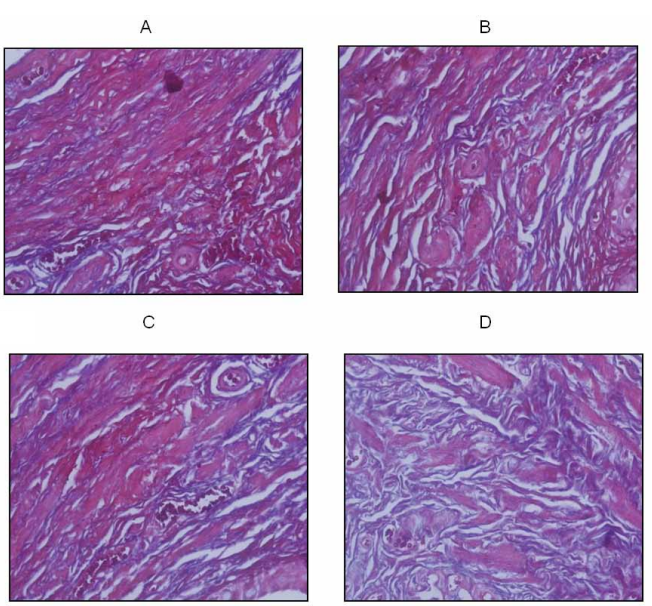


	Control	PO2	PO4	PO8	PO12
Muscle	70.85%	58.25%*	49.76%*	48.20%*	48.80%*
Collagen, vessels and elastin	29.15%	41.75%*	50.24%*	51.80%*	51.20%*

PO = post operative in each timepoint - 2, 4, 8 and 12 weeks.

* Kruskal Wallis – $p< 0.05$ – compared with control group.

Figure 3 - Masson´s Tricrome staining at the different time points (Magnification = 400X).



A = Control Group at 8 weeks; B =2 weeks; C = 4 weeks; D = 8 weeks after urethrolysis.

Stained in red = muscle.

Stained in purple = collagen and elastin.

determined by a positive sneeze test performed 4 weeks following vaginal distension, only one third of the rats were incontinent (15). In the present study, we observed an easily reproducible periurethral lesion. Once we standardized the periurethral lesion technique, all animals developed a significant and consistent decrease in LPP.

Another method described to determine urethral resistance in rodents is the tilting table test (18). The rat is mounted on a vertical tilting table to simulate the orientation of the human pelvis with respect to gravity and the spinal cord of the rat is transected at the T8-T9 level to eliminate bladder interference, while maintaining the urethral closure mechanisms intact (18).

Other studies with female rats were described injuring the pudendal nerve (19). The main problem with pudendal nerve crush models is that they create a recoverable localized denervation of the striated muscle component and the whole pudendal innervation. To study stem cells effect on urethra, the urethral lesions should not heal spontaneously, because we need long term follow-up. In addition, it should include the striated and smooth urethral sphincter component to better reproduce the clinical aspects.

In 2004, an animal model of intrinsic sphincter dysfunction has been described by electrocoagulation of the urethral surrounding tissues (20); this model created a long lasting decrease in urethral resistance and more significant tissue damage than childbirth simulations. Another study developed a urethral damage by performing a transabdominal urethrolysis in female rats. The authors observed a significant decrease in LPP and retrograde urethral perfusion pressure up to 24 weeks. An histological analysis demonstrated a reduction of muscle fibers and an increase of the conjunctive tissue (10). In our study, we performed a similar intervention in rabbits confirming that such intervention can consistently decrease LPP associated with histological urethral changes.

Few studies were performed in rabbits in order to develop a urinary incontinence animal model. In a work with male rabbits, after sectioning the pudendal nerve bilaterally, it was observed a decrease in urethral resistance. However, after 12 weeks, there was a recovery of the urethral occlusion pressure. The authors hypothesized a probable spontaneous regeneration of the nervous tissue (21).

The main reason to develop an animal model is to allow safety tests before human use. In the past years, it has been proposed cell therapy injection as an alternative for urinary incontinence treatment. Human use has been described based mainly on tests performed in isogenic or imunocompromised rats (9). We believe that such therapy should be tested using autologous cell transplantation before human use. The lack of urinary incontinence animal model that allows autologous cell harvesting restricts such tests. Our animal model can test autologous cellular therapy injection in the urethra wall.

The major difficulty to create a urinary incontinent animal model is related to functional-anatomic characteristics that distinguish man from other animals. This difficulty in getting an animal model has led to surgical approaches and therapies for humans without being suitably tested in animal trials. The mostly used urinary incontinence and sphincter deficiency animal models to test injectable cell therapy have been developed in female rats. The main problems with the use of such animals are: the difficulty to perform autologous tests, the high regenerative capacity leading to recovery of the urethral function after a certain period of time, the small bladder capacity (around 1.5 mL), difficulty to determine the leak pressure due to bladder interference (bladder contraction during functional evaluation), a relatively longer urethral length with a small diameter and thin wall.

Rabbits present reasonable anesthetic-surgical resistance, allowing multiple surgical interventions and autologous cell harvesting; present a urethral wall with good thickness, allowing reproductible and precise injections during cell therapy tests. We found that female New Zealand rabbits present a hypospadic urethral meatus. Thus, the urethral catheterization is an extreme challenging job. We designed a vaginoplasty in order to reach the meatus. Nevertheless, the urethral catheterization still was challenging and sometimes it could not be reproducible. We also performed a suprapubic vesical catheterization trying to accomplish

the cystosmetic evaluation and LPP determination. However, it changed the bladder behavior, created inflammation on the puncture spot and generated bladder leakage during LPP evaluation. One can criticize an animal model in male rabbits, as the majority of patients with stress urinary incontinence are women. However, we were looking for a reproducible animal model that would allow testing safely and efficiently autologous cells injections into the urethra wall. Furthermore, it has been described autologous muscle derived from stem cells in the human male urethra, without an extensive animal study (6).

Despite interesting initial clinical outcomes in SUI treatment using cell therapy, we need more basic research to clearly demonstrate safety for human use. The potential risks, such as teratogenicity, cell migration and mutagenicity should be better evaluated, especially when treating a benign, non-life threating disease. The present animal model is a new alternative to test safely and effectively cell therapy to treat stress urinary incontinence. It also has the possibility to test autologous cell therapy as one alternative to functional and anatomical regeneration in a urethral intrinsic sphincter deficiency animal model.

CONCLUSIONS

Transabdominal urethrolysis created sphincteric insufficiency in rabbits with significant decrease in the LPP associated with decrease of smooth muscle fibers and increase of collagen density. This animal model can be used to test autologous cell therapy for stress urinary incontinence treatment.

CONFLICT OF INTEREST

None declared.

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