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Original Article

Micro-recanalization in a biodegradable graft for reconstruction of the vas deferens is enhanced by sildenafil citrate

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Abstract

This study investigated the effect of sildenafil citrate on micro-recanalization and neovascularization, which were previously demonstrated in a rat model using biodegradable grafts (BGs) for vas deferens reconstruction. A total of 24 male rats underwent bilateral vasectomy with removal of a 0.5-cm vasal segment and were randomly assigned to four groups. Groups 1 and 2 underwent immediate vasovasostomy. Groups 3 and 4 underwent interposition of a 0.5-cm BG in the vasal gap. Groups 1 and 3 were given 5 mg kg^{-1} day^{-1} oral sildenafil. Other groups were given placebo. Rats were housed with females 12 weeks postoperatively. Reconstructed vasal segments were harvested 16 weeks postoperatively and analyzed histologically. Fluid from the distal vasal stump was analyzed for motile sperm. Urine samples obtained 16 weeks postoperatively were analyzed for cGMP levels. cGMP levels in rats treated with sildenafil were significantly higher than in control rats. No pregnancies were sired by grafted groups. In all, 5/6 rats in group 1 and 3/6 rats in group 2 sired litters. No motile sperm were noted in the vasal fluid of the grafted groups. Motile sperm were noted in all rats in group 1 and in 5/6 rats in group 2. In addition, 29 and 4 microcanals were detected in the sildenafil and placebo groups, respectively ($P = 0.023$). No microcanal exceeded 3 mm in length. An average of 12 and 28 blood vessels per graft were noted in the placebo and sildenafil groups, respectively ($P < 0.0001$). In conclusion, sildenafil enhances micro-recanalization and neovascularization in BG used for vas deferens reconstruction, but does not increase the microcanal length after 16 weeks.

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1 Introduction

Obstructive azoospermia (OA) is a significant entity in male infertility, estimated to account for up to 40% of azoospermia cases. Obstruction may be present at various levels along the course of the male reproductive tract, including the epididymis, the vas deferens and the ejaculatory ducts. OA may be secondary to congenital disorders, such as congenital bilateral absence of the vas deferens, commonly associated with cystic fibrosis, as well as to partial vasal absence. Obstructive lesions leading to azoospermia may be acquired, secondary to scrotal or pelvic trauma, surgery and infection, causing fibrosis, scarring or frank disruption of the male genital tract. The intentional interruption of the vas deferens during vasectomy is one of the most common causes for OA in the United States. Bilateral vasectomy is performed

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on nearly 500 000 men every year in the United States [1]. Vasectomy reversal is performed in nearly 6% of men who had a vasectomy [2]. Furthermore, approximately 6% of men who suffer iatrogenic injury to the vas deferens require reconstruction [3].

The aim of reconstructive surgery for OA is to regain continuity of the obstructed male genital tract, thus allowing for reappearance of sperm in the ejaculate and, eventually, achievement of natural conception. The preferred reconstructive technique involves the identification and isolation of the obstructed site, and resection of the obstructed segment followed by primary anastomosis of the edges of the genital tract on both sides of the obstructed segment, after confirming the patency of the tract distal and proximal to the obstruction. However, the presence of long obstructed or missing segments might preclude the accomplishment of such an anastomosis, or put it under tension that could cause disruption or leakage.

Tissue engineering strategies using biodegradable polymer scaffolds have been studied for regeneration of various tissues and organs such as skin, cartilage, liver and peripheral nerves. We have previously explored the feasibility of adapting this concept for treatment of OA, and investigated the possible use of biodegradable polymer grafts (Figure 1) in reconstructive procedures involving long obstructed or missing segments of the male reproductive tract [4]. We were able to demonstrate early evidence of post-vasectomy micro-recanalization through plain biodegradable grafts (BGs) that were devoid of any chemical or biological cues.

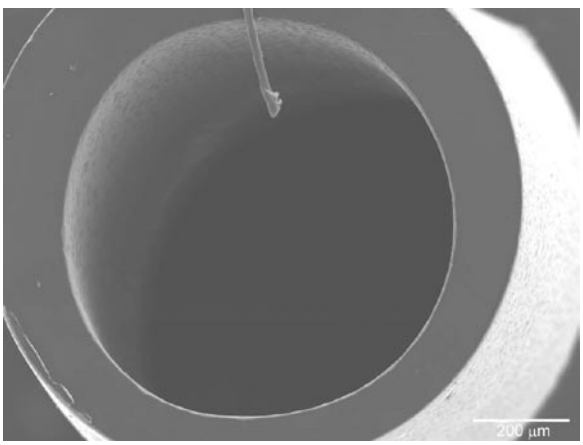

Figure 1. Scanning electron microscope image of a polymer graft [4].

These microcanals may be similar to those described by Freund *et al*. [5] in previous vasectomy sites removed at the time of vasovasostomies.

Sildenafil citrate is a phosphodiesterase inhibitor that was initially developed as an anti-hypertensive and anti-angina agent. Clinical trials showed little effect on angina but the drug induced penile erections and has subsequently been marketed for erectile dysfunction (ED). Sildenafil is a selective inhibitor of cGMP-specific phosphodiesterase type 5. In addition to mediating relaxation of the corporal smooth muscle, cGMP is involved in many other cellular signaling pathways mediated by cGMP-gated ion channels and cGMP-dependent protein kinases. cGMP has been implicated in post-natal angiogenesis. Sildenafil citrate leads to increased vascular endothelial growth factor expression and angiogenesis in a rat model of coronary ischemia [6]. In addition, administration of oral sildenafil citrate improved recovery of spontaneous 'normal' erections following radical prostatectomy in a controlled study [7]. It was postulated that this effect may be the result of improved endothelial function combined with neuronal regeneration and neuroprotection. Sildenafil also increases brain levels of cGMP, evokes neurogenesis and reduces neurological deficits when given to rats 2 or 24 h after stroke [8].

We therefore hypothesized that administration of sildenafil citrate would lead to enhanced neovascularity and microcanal formation in implanted biodegradable vas deferens grafts.

2 Materials and methods

The BGs used in this study were designed and constructed at Iowa State University (Ames, IA, USA). Porous conduits for grafting were made of PDLA (DURECT, Birmingham Polymers [Pelham, AL] Lactel Poly-DL-lactide, inherent viscosity 63 mL g^{-1} in $CHCl_3$ at 30 ºC). Poly-DL-lactide was dissolved in chloroform at 30% w/v. Sodium chloride was ground in a mortar and pestle and sieved through a #140 (106 μm) mesh. Sieved NaCl was added to the polymer/chloroform solution to obtain finished conduits with 75% porosity. Polyvinyl alcohol-coated glass capillary tubes were dipped into the PDLA/salt suspension. Tubes were soaked in water to remove NaCl and polyvinyl alcohol and conduits were dried in a desiccator. Desiccated conduits were stored in sampling tubes at 4 ºC.

Micropatterned PDLA films were inserted into each

conduit with the micropatterned luminaly exposed. Four-inch micropatterned silicon casts were used to make the micropatterned films. The micropattern was fabricated on wafers using ultraviolet lithography and reactive ion etching as described by Recknor *et al*. [9]. The micropattern consisted of parallel ridges with height, thickness and separation of 4, 14 and 16 μm, respectively. Wafers were spin-coated with 5% w/v polyvinyl alcohol dissolved in water and then the 30% PDLA solution, allowing sufficient drying time after coatings. Wafers were submerged in 18 mΩ filtered water (Nanopure; Thermo Scientific, Waltham, MA, USA) to dissolve the PVA layer and detach films from the wafer. Sections were then cut out of the film, rolled up against a capillary tube and inserted into the conduits with the pattern aligned longitudinally with the conduits.

The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Iowa. A total of 24 male Sprague–Dawley rats were used. Rat age at arrival was 2 months with a weight range of 225–249 g (Harlan Laboratories Inc., Indianapolis, IN, USA).

Rats were randomly assigned to four groups with six in each group. All animals underwent bilateral vasectomy under general anesthesia with intraperitoneal sodium pentobarbital through a low midline abdominal incision. A 0.5-mm segment of vas was removed on both sides. Groups 1 and 2 underwent immediate end-to-end vasovasostomy with 10-0 nylon suture. Groups 3 and 4 underwent microsurgical interposition of a 0.5-mm PDLA graft in end-to-end fashion to re-approximate the vas deferens with 10-0 nylon suture.

Groups 1 and 3 were given 5 mg kg^{-1} daily oral sildenafil citrate (mixed into peanut butter balls) for 16 weeks while groups 2 and 4 were given placebo. Twelve weeks after vasectomy and reconstruction, each male rat was housed with a female rat for a period of 10 days, which corresponds to about two estrous cycles of the female rat. If pregnancy did not occur, the female rat was transferred to the next male's cage.

At 16 weeks after vasectomy and reconstruction, a urine sample was obtained by suprapubic aspiration and immediately frozen on a dry ice and 95% ethanol slurry for later analysis. The reconstructed segments of vas were harvested and placed in formalin. A sample of vasal fluid from the abdominal end of the vas was obtained and mixed with one drop of phosphate-buffered saline for immediate identification of motile sperm by light microscopy. The animals were then killed by lethal intraperitoneal injection of pentobarbital and confirmed dead by thoracotomy.

Rat urine samples were analyzed for cGMP levels by enzyme-linked immunosorbant assay in accordance with the manufacturer's protocol (Cell Biolabs, San Diego, CA, USA). This cGMP assay is designed to measure cGMP in cell culture supernatants, plasma, serum, saliva, urine and cell lysates. The kit selectively measures cGMP levels without any significant cross-reactivities to other nucleotides or cyclic nucleotides. The kit has a detection range of 1 to 1 000 pmol mL^{-1} cGMP.

Harvested PDLA grafts were serially sectioned at 1-mm intervals and stained with hematoxylin and eosin (H&E) for microscopic analysis by a single pathologist who was blinded to the treatment groups. Representative sections were also stained for CD31 (PECAM-1) for definitive identification and quantification of endothelial-lined vessels. The final count of these vessels was calculated by averaging two separate countings at least 24 h apart.

The rats were housed in horizontal Thoren (Hazleton, PA, USA) 12″ × 12″ #4 Udel cages maintained at 22.2 ºC. One rat was housed per cage unless breeding, at which time one male and one female were housed together. Bedding was SoftZorb paper-based laboratory bedding (Northeastern Product Corp., Warrensburg, NY, USA). Feed was NIH-31 modified 6% mouse/rat sterilizable diet #4913. Water was filtered. Statistical analysis was performed using the Wilcoxon rank-sum test with $P < 0.05$ considered as statistically significant.

3 Results

cGMP levels in rats treated with 16 weeks of oral sildenafil citrate were significantly higher compared with the placebo group (Wilcoxon rank-sum test, $P = 0.008$). The sildenafil group had a median cGMP level of 191.5 pmol mL^{-1} compared with 59.0 pmol mL^{-1} in the placebo group (Table 1).

No pregnancies were sired by any rat with implanted PDLA grafts. Five of six rats in the primary vasovasostomy group were treated with sildenafil sired litters and three

Table 1. Distribution of cGMP levels (pmol mL^{-1}) in rats treated with 16 weeks of oral sildenafil citrate *vs.* controls.

Treatment	*N*	Median	25th percentile	75th percentile
Placebo	6	59.0	32.0	78.0
Sildenafil	6	191.5	188.0	223.0

of six rats in the primary vaso-vasostomy group were treated with placebo sired litters.

No motile sperm was observed in the distal vasal fluid of rats in the grafted groups. Motile sperm was present in six of six rats in the sildenafil group and in five of six rats in the placebo group.

At 16 weeks, all PDLA grafts were easily identifiable, grossly intact and sealed to the vas at both ends. Histological examination of the PDLA grafts revealed the presence of a granulomatous inflammatory response within the lumens. Also identified were multiple microcanals lined with a distinct layer of cuboidal epithelial cells within the lumens of the grafts. A total of 29 microcanals were identified in the sildenafil groups and four microcanals were identified in the placebo groups. The average microcanal length was 2 mm. None of the 33 microcanals exceeded 3 mm in length. Wilcoxon rank-sum test showed a significantly greater number of microcanals in the sildenafil group compared with placebo ($P = 0.023$). Of the 12 rats in the sildenafil group, there were 6 (50%) that had two or more microcanals and four (33%) with no microcanals. In the placebo group, there was only one (17%) that had two or more microcanals and nine (75%) with no microcanals. Similar results were found if type of vasectomy was accounted for in the analysis (Cochran–Mantel–Haenszel statistic based on rank scores, $P = 0.014$). In the sildenafil group, two or more microcanals were found in four of six (67%) that had vasovasostomy and two of six (33%) that had PDLA grafts. In contrast, in the placebo group, only one of six (17%) in those with vasovasostomy and none of six in those with PDLA grafts had two or more microcanals (Table 2).

A large number of small blood vessels were identified on H&E staining throughout the walls and lumens of the PDLA grafts.

CD31 staining of the PDLA grafts confirmed the presence of endothelial-lined vessels in their walls and lumens. Wilcoxon rank-sum test showed a significantly greater number of stained vessels in the sildenafil group compared with placebo ($P < 0.0001$). The median number of stained vessels in the sildenafil group was 29 compared with 11.5 in the placebo group. Similar results were found if type of vasectomy was accounted for in the analysis (Cochran–Mantel–Haenszel statistic based on rank scores, $P < 0.0001$) (Table 3).

4 Discussion

Reconstruction of the vas deferens in the setting of a long segment of obstruction remains a challenge when primary repair is not an option. We previously demonstrated that reconstruction of the vas with a

Table 2. Comparison of the number of microcanals between placebo and sildenafil.

Number of canals	Placebo, *n* (%)			Sildenafil, *n* (%)			*P*-value
	Vasovasostomy	PDLA	All	Vasovasostomy	DLA	All	
0	4 (67)	5 (83)	9 (75)	1 (17)	3 (50)	4 (33)	0.014[a]
1	1 (17)	1 (17)	2 (17)	1 (17)	1 (17)	2 (17)	
2	1 (17)	0 (0)	1 (17)	0 (0)	2 (33)	2 (17)	
> 2	0 (0)	0 (0)	0 (0)	4 (67)	0 (0)	4 (33)	

Abbreviation: PDLA, poly-D-lactide.
[a]Cohran–Mantel–Haenszel statistic based on rank scores, adjusting for type of vasectomy.

Table 3. Comparison of the number of CD31-stained vessels between placebo and sildenafil groups.

Type of vasectomy	Placebo		Sildenafil		*P*-value
	Median	25th–75th percentile	Median	25th–75th percentile	
Vasovasostomy	11.5	11–14	28.5	27–34	< 0.0001[a]
PDLA	11	10–12	29.5	23–32	
All	11.5	10.0–12.5	29	25–33	< 0.0001[b]

Abbreviation: PDLA, poly-D-lactide.
[a]Cohran–Mantel–Haenszel statistic based on rank scores, adjusting for type of vasectomy. [b]Wilcoxon rank-sum test.

biodegradable PDLA graft in a rat model led to some cases of microrecanalization as early as 12 weeks after implantation [4]. All cases showed evidence of neovascularization. We therefore sought to improve neovascularization of the PDLA graft with the aim of increasing the rate of microcanal formation.

As a type 5 phosphodiesterase inhibitor, sildenafil citrate has been marketed as a successful agent in the treatment of both ED and pulmonary hypertension. Its mechanism of action involves inhibition of the breakdown of cyclic GMP, which in turn leads to local vasodilation. Recently, sildenafil has also been shown to increase expression of vascular endothelial growth factor and angiopoietin-1, leading to increased neovascularization in a rat model of coronary ischemia and promoting functional recovery after stroke in a rat model of cerebral ischemia [6, 8].

Although the current study demonstrated increased neovascularity within PDLA grafts in the sildenafil group as well as a significantly increased number of microcanals, none of these canals extended the length of the grafts, no motile sperm were observed in smears from the distal ends of the grafts and none of the grafted animals were able to achieve a pregnancy, which is the ultimate outcome of a successful reconstruction of an obstructed vas deferens. This is likely secondary to obliteration of the graft lumen by an intense inflammatory reaction, which is only later followed by neovascularization and ultimately, recanalization. Sixteen weeks may simply not be a sufficient time period to allow this process to extend the length of the 0.5-cm graft despite the use of sildenafil. A functional obstruction may also exist within the lumen of the graft, as this segment lacks smooth muscle and is therefore not capable of peristalsis.

Future studies will be necessary to investigate the effect of a longer period of time after graft implantation on the patency of the PDLA graft, the extent of micro-recanalization and the ability to achieve pregnancy following this type of reconstruction. Studies are also planned to investigate whether the addition of selected growth factors to the grafts in combination with systemic sildenafil could enhance microcanal formation and length. Finally, the fact that the sildenafil citrate induced an increase in post-vasectomy neovascularity and micro-recanalization, which has been demonstrated in this study, raises the question of whether the use of this medication after vasovasostomy or vasoepididymostomy in human subjects could lead to improved patency and pregnancy outcomes of these procedures. Investigation of this interesting possibility is beyond the scope of this study, and would require separate future research.

In conclusion, sildenafil citrate stimulates neovascularity and the formation of microcanals in PDLA grafts used to reconstruct the vas deferens in a rat model but does not increase microcanal length after 16 weeks.

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