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BioSphincter a Regenerative Medicine Approach to Treat FI

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Abstract

A healthy sphincter physiology is a complex interplay between neural and muscle population, responsible for relaxation and contraction, which allow feces to pass and reestablishment of closure. The loss of integrity of neuromuscular functionality or cellular component results in fecal incontinence (FI). The current available treatments have been disappointing in long-term relief. This chapter represents a regenerative medicine approach to this debilitating disease, wherein a new internal anal sphincter (IAS) BioSphincter™ is bioengineered from the patient's own cells and implanted. It results in long-term restoration of the cellular integrity and reinstatement of the physiological function of the IAS. Following implantation in rodents, the engineered sphincters became vascularized and maintained their phenotype and functionality. The developed IAS BioSphincter™ were validated to treat the FI in large animals and successfully restored anorectal functionality. According to NIH/NIDDK, one out of seven people report to health care providers complaining of fecal incontinence. This chapter elucidates the long road in developing on implantable bioengineered IAS "BioSphincter™" that would benefit and improve the quality of life of a large socially distressed segment of the population.

Keywords: fecal incontinence, regenerative medicine, BioSphincters, neural progenitor cells, anorectal physiology

1. Introduction

A healthy anorectal functionality is a coordinated interplay between the enteric nervous system, smooth muscle of internal anal sphincter (IAS), striated external anal sphincter and puborectalis muscles [1]. Anomalies in any of this individual or group of tissues may lead to anorectal irregularities and diseases [2]. Fecal incontinence (FI) is devastating from a hygiene perspective due to involuntary soiling of liquid and solid stool and results in the distressing psychosocial impact on the patient [3]. Injury to the perineum may also result in the complete or partial destruction of the anal sphincter and distal rectum potentially resulting in persistent incontinence [4]. The resulting psychological stress, social stigma, decreased self-esteem and productivity can be overwhelming. In the USA, men and women suffer from FI equally with a range of 2–6% in people aged 20–30 years. The prevalence increases to over 15% in people older than 70 years [5].

Clinical characteristics of FI have been correlated with underlying sphincter pathology [1]. In the classical FI, the pelvic floor muscles are dysfunctional (due to muscle or nerve damage) and result in the frequent urge of incontinence. The urge of incontinence is mainly due to external anal sphincter defects and lower anorectal

squeeze pressures. Patients with the urge of incontinence have FI episodes with awareness of the event but cannot prevent it because of the inability to increase anorectal pressures [1, 6, 7].

The passive FI caused an isolated or combined loss of smooth muscle function (IAS), skeletal muscle function (EAS), anorectal sensory mechanisms or neural control [8, 9]. It leads to loss of the sense that rectum is full and results in unknowingly leakage of stools, mucus, and flatus. Passive incontinence occurs without the patient's awareness of the event until after incontinence has occurred [6]. Patients with passive incontinence are more likely to have internal anal sphincter defects and lower anorectal resting pressures. The anal resting tone is produced by the internal anal sphincter (IAS) and the external anal sphincter. The IAS contributes 60–70% of the anal tone [10]. In addition, patients with passive incontinence have been shown to have more frequent and exaggerated IAS relaxation compared to continent controls [11]. Patients with FI have been shown to have variable loss of the Recto Anal Inhibitory Reflex (RAIR) [12].

Currently, there is no satisfactory long-term treatment for FI. Epidemiological studies indicated that most patients suffering from FI do not consult to clinicians and depend on self-management or rely on the use of adult diapers. The classical treatment of FI becomes more involved in accordance with the extent and severity of the incidences of incontinence.

Conservative management of FI is usually initiated with educating the patients with behavioral techniques. These techniques such as scheduling toileting and preventive strategies [13]. The next step is the incorporation of dietary changes using fiber supplements or laxative to normalize stool consistency [14]. Along with dietary modulations, antidiarrheal drugs, alpha (1 and 2) receptor agonists could also be used to control the frequency of FI episodes [15]. Pelvic floor muscle exercise and biofeedback are other conservative methods to manage initial stages of FI. Biofeedback methods are behavioral management that incorporates electronic and mechanical devices to emphasize bowel and muscle retraining. Pelvic floor muscle exercises with biofeedback improve sense and strength of pelvic floor muscles for contraction during rectal distention and uncontrollable urge of FI [16, 17]. According to an observational study, these conservative methods resulted in 50% reduction in the frequency of FI and 21% adequate relief in FI [13]. The effectiveness and success of these measures may help in the management of mild cases of FI.

If the patient does not improve with the mentioned conservative methods, the patient is offered advanced therapies. Advanced therapies are more invasive and involve different levels of surgical interventions such as electrical stimulation, sphincteroplasty, injection of bulking agents, and implantable devices. Sacral nerve and tibial nerve stimulation found to be more effective than electrical stimulation of muscles [18–20]. In a randomized controlled trial on patients with structurally intact and innervated sphincters, the implantation of a battery-operated stimulator was found to be effective from 36 to 50% [18, 19]. The frequency of episodes of FI was reduced during stimulation, but unaffected without stimulation or similar to sham [13]. The implantable devices such as artificial bowel sphincter [21], magnetic beads [22] and synthetic polymer rings are implanted around the anal canal to augment the pressure. There is a lack of randomized controlled trial towards long-term safety and efficacy of these procedures. The sphincteroplasties (suturing of the separated sphincter) and graciloplasty (wrapping of gracilis muscle around the anal canal) are another class of surgical procedures to treat FI. These procedures have shown varying rates of success and high chances of obstructed defecation. Inert materials (silicone elastomers, ceramic beads) or biopolymers (polycaprolactam beads) as bulking agents injected around the anal canal to increase resting pressure [23]. There was no specific success reported regarding long-term efficacy.

However, a 3-month follow up study of injection of dextranomer microspheres resulted in a 50% reduction in FI frequency in 52% patients [24].

Cell delivery is advanced translation method for long-term efficacy in FI. Stem cell constructs were developed, and were able to generate smooth muscle tone but lacked innervation [25]. Autologous transplantation of muscle progenitor cells into the sphincters exhibited potentials for re-stabilization of myogenic functionality in the anal sphincters [26]. Delivery of autologous human adipose-derived stem cells in poorly functioning sphincter muscle as replacement of fibrous tissues acted as a mechanical support for physiological functions [27]. Injection of autologous myoblasts into the external anal sphincter defect also resulted as a safe and promising approach to improve symptoms of FI induced owing to obstetric anal sphincter trauma [28]. Sphincters are complex organs for cell delivery. There are several challenges to overcome in direct cell delivery, such as specific types and dosages of cells, circular distribution and orientation of cells around the anal canal after injection, functional integration with host cells and long-term effects such as biodistribution, tumorigenicity.

Current cell delivery technologies focus either on the reinstatement of the striated muscle of the external anal sphincter or mechanical support to the sphincter, with little attention on the reinstatement of IAS function [29–32]. The terminal gut function requires coordinated contraction and relaxation of the smooth muscle of rectum mediated through the enteric nervous system of IAS [2, 6]. To remedy an injured anus, it is imperative to reinstate both smooth muscle and intrinsic neural components of IAS. We describe the evolution of a regenerative medicine approach proposed to provide critical components to reinstate function in the anorectum and remedy passive fecal incontinence caused by injury to the IAS. According to this hypothesis, implantation of engineered autologous BioSphincters reinstate IAS function and restore fecal continence. Autologous smooth muscle and neural progenitor cells from gut biopsies were used to bioengineer intrinsically innervated IAS [33, 34]. Autologous functional intrinsically innervated IAS construct was successfully implanted into healthy animal models. Following implantation in rodents, the engineered sphincters became vascularized and maintained their phenotype and functionality [35–38]. A large animal model of passive fecal incontinence was developed and demonstrated sustained restoration of fecal continence, and restoration of basal tone and restoration of RAIR after implantation of engineered autologous, intrinsically innervated internal anal sphincter (IAS) BioSphincters [10, 39] (Figure 1).

Figure 1.
Regenerative medicine approach to treat fecal incontinence using autologous bioengineered BioSphincter.

This chapter summarizes the regenerative medicine approach of bioengineering of BioSphincters, including developmental stages of the technology, challenges, process optimization, characterization, detail pre-clinical evaluation of the BioSphincter towards the treatment of FI.

This chapter encompasses both in vitro and in vivo studies designed to support the safety and efficacy of bioengineered sphincters. Studies performed in vitro include the generation of three-dimensional internal anal sphincter models using rabbit IAS smooth muscle cells and human IAS smooth muscle cells. The in vitro studies also describe the intrinsic innervation of bioengineered IAS sphincters. Studies performed in vivo are described in two parts, small animal rodent studies and a large animal, rabbit fecal incontinent model. Small animal rodent studies included: (1) generation and implantation of IAS smooth muscle cell sphincter into a C57BL/6 J rodent; (2) generation and implantation of human innervated bioengineered sphincters into an athymic rodent model, at subcutaneous and peri-anal sites. Large animal studies demonstrating successful implantation of intrinsically innervated autologous IAS BioSphincters were conducted in a rabbit model of fecal incontinent.

2. Bioengineering an in vitro three-dimensional physiological model of the internal anal sphincter from rabbit smooth muscle cells

The objective of the early studies was to develop an in-vitro three-dimensional (3-D) physiological model of the IAS smooth muscle cells. In this initial attempt, rabbit origin IAS smooth muscles were cultured on top of a loose fibrin gel; subsequently, these cells migrated and self-assembled in circumferential alignment. As the cells matured, the fibrin gel contracted around a 5-mm-diameter silicon mold, resulting in a 3-D cylindrical ring of sphincteric tissue [40].

Histological analysis exhibited a gradient of cell alignment in the bioengineered IAS sphincters. The engineered sphincters were analyzed for physiological functionality using an isometric force transducer. Constructs were placed between a stationary central pin and the measuring arm of the organ bath transducer (Harvard Apparatus, Holliston, MA). The bioengineered sphincter generated a spontaneous basal tone, and treatment with 8-bromo-cAMP (8-Br-cAMP) resulted in relaxation. In the next step, agonist-induced stimulation (using acetylcholine) resulted in calcium- and concentration-dependent peak contraction. This effect was diminished by the addition of 8-Br-cAMP. Similar bioengineered IAS sphincters were also generated using colonic smooth muscle cells. IAS constructs display significant differences in functionality compared to colonic smooth muscle cells constructs, which confirmed tissue specificity and functionally to IAS [40].

This was the first successful attempt to develop 3-D in vitro model of engineered IAS sphincters using smooth muscle cells of IAS. Bioengineered IAS sphincters displayed circular cell alignment and physiological functionality. The functionality and physiological response in engineered tissues exhibited similarity to IAS smooth muscle in vivo [38].

3. In vivo cytocompatibility and functionality analysis on subcutaneous implantation of physiologically functional bioengineered internal anal sphincter

After successful bioengineering an IAS specific sphincter tissues, the next goal was to evaluate the in vivo biocompatibility and adverse reaction. The objective of these studies was to test the post-implantation functionality of bioengineered sphincters engineered using IAS smooth muscle cells. **Table 1** summarized the detail study design.

In this endeavor, smooth muscle cells were isolated from the IAS of donor C57BL/6 mice. Smooth muscle cell constructs were engineered on Sylgard coated plates using fibrin gel, as described previously [40]. The engineered constructs were successfully implanted into the subcutaneous region of same strain mice and treated with either fibroblastic growth factor-2 or saline as controls using a micro-osmotic pump. Mice were euthanized after 4 weeks, and the implant was harvested. The implant was intact, healthy in color without any degradation, and interestingly displayed muscle attachment to the back of the mouse, with neovascularization. Constructs exhibited no external sign of inflammation, fibrosis, or infection, because of the use of syngeneic tissue. The supplement of FGF-2 also helped in tissue viability, cellular integrity, and vascularization. The harvested tissues maintained smooth muscle alignment and phenotype [37, 38].

The post-implant harvested constructs were analyzed for force generation. The harvested implants generated and maintained the spontaneous basal tone in the absence of any external stimuli. The developed tone confirmed the integrity of ionic membrane characteristics, membrane receptors and their intracellular signaling mechanisms for contraction and relaxation. On treatment of a relaxing stimulant such as a vasoactive intestinal peptide (VIP), the force and magnitude of relaxation were consistent before and after implantation. The rapid, and dose-dependent sustained (over 30 min without signs of muscle fatigue) contractions on the treatment of acetylcholine and phorbol dibutyrate was elicited as well. The physiological studies confirmed that implanted bioengineered sphincters maintain IAS physiological functionality after implantation [37, 38].

In summary, IAS sphincters using smooth muscle tissue could be bioengineered. The bioengineered sphincters were cytocompatibility, functional, without any adverse reaction and had potential to be used as a graft for dysfunctional internal anal sphincter [37, 38].

Steps	Study objective(s)	Test article	Animal model	Key outcome (e.g., safety (tumor/tox/biodistribution), efficacy, characterization, stability, degradation)
<i>Study purpose: cell isolation</i>				
Isolation of SMC	To isolate IAS smooth muscle cells (SMC) and characterization of smooth muscle	In vitro expanded IAS smooth muscle cells	C57BL/6J mice	Smooth muscle cells expressed cell lineage appropriate phenotype markers
<i>Study purpose: bioengineered sphincters</i>				
Bioengineering sphincters with smooth muscle	Characterize the bioengineered sphincters	Bioengineered sphincters	C57BL/6J mice	Formation of stable sphincters
<i>Study purpose: implantation of bioengineered sphincters into rodent</i>				
Implantation of bioengineered sphincters	Optimization of the implantation procedure	Bioengineered sphincters	C57BL/6J mice	Bioengineered sphincters were implanted subcutaneously on syngeneic mice (C57BL/6J) model
<i>Study purpose: end points analysis</i>				
Bioengineered sphincters histopathology	Analysis of fibrosis/inflammation and functional activity	Implanted bioengineered sphincters	C57BL/6J mice	No fibrosis or inflammation was observed in bioengineered sphincter implants

Table 1.
Summary of nonclinical study for safety and efficacy of bioengineered sphincters in C57BL/6J mice.

4. Bioengineering an internal anal sphincter derived from smooth muscle cells isolated from the human internal anal sphincter

The preliminary work in the previous sections using SMCs harvested from animal models confirmed the feasibility of engineering functional physiologic IAS constructs and initial biocompatibility. [40]. The next objective was to validate the feasibility of engineering IAS sphincter constructs from SMCs of human IAS origin.

Human IAS was received from NDRI and SMCs were harvested following previously described protocol. At confluency, SMCs were seeded on Sylgard coated plates with fibrin gel. Cells migrated and aligned circularly around the Sylgard mold located at the center of the plate. All the 3-D bioengineered sphincter constructs successfully formed within 5–10 days of seeding of Human IAS SMCs [34].

The developed human IAS constructs displayed the essential characteristics of a native functional IAS; the bioengineered IAS constructs able to generate the spontaneous myogenic basal tone and respond to different pharmacological agents. Bioengineered human IAS sphincters also exhibited dose-dependent force generation in response to different stimulants. The IAS smooth muscle constructs displayed a tissue-specific basal tone compared to colonic muscle cells. The basal tone, acetylcholine-induced contraction and PdBU generated were reduced by calphostin-C but not with Y-27632. The detailed functionality resulted that the protein kinase C (PKC) pathway (independent of the Rho/ROCK pathway) appeared to be responsible for IAS specific tone and contractions [34].

The process of bioengineering IAS constructs using human IAS smooth muscles was highly reproducible. The developed IAS muscle constructs were functionally similar to native IAS sphincters. This was the first report demonstrating the generation of a functional in vitro model of human IAS that may be used for the elucidation of mechanisms associated with smooth muscle sphincter myogenic malfunction and for the investigation of treatments for fecal incontinence [34].

5. Bioengineered IAS generated from human cells and preliminary biocompatibility and functional analysis after implantation in an athymic rodent model

In the previous sections, IAS muscle constructs were successfully bioengineered with animal and human origin IAS circular muscles. The bioengineered mouse IAS muscle constructs displayed physiological functionality after implantation in wild type mice. However, compare to anatomy and physiology of native IAS sphincters, the bioengineered muscle constructs lacked innervation of the neuronal population. Therefore, the next target in these studies was to intrinsically innervation of bioengineered IAS muscle constructs and evaluation of cellular viability, physiological functionality, and safety after implantation. **Table 2** summarized the detail study design.

In this effort, the human IAS muscles were harvested and cultured as described previously. The neuronal cell line was isolated from a D13 embryo from H-2Kb-tsA58 immortomouse. The bioengineering of constructs was divided into two steps. In the first step, the isolated neuronal stem cells were mixed with hydrogel and plated in the Sylgard coated plates. After gelation, IAS origin smooth muscle cells were mixed with the collagen gel and overlaid to the previous cell-hydrogel. A fully compacted sphincter-like construct were developed in the first 60 h [35].

The neuronal stem cells differentiation towards functional neurons was carried out in a specific media targeted to neural differentiation. The bioengineering

Steps	Study objective (s)	Test article	Animal model	Key outcome (e.g., safety (tumor/tox/ biodistribution), efficacy, characterization, stability, degradation)
<i>Study purpose: cell isolation</i>				
Isolation of SMC	To isolate IAS smooth muscle cells (SMC) and characterization	In vitro expanded IAS smooth muscle cells	Cadaver human	Smooth muscle cells expressed cell lineage appropriate phenotype markers
Isolation of neural cells from embryo of immortomouse	To isolate neural cells and characterization	In vitro expanded neural cells	h-2kb-tsA58 immortomouse	Neural cells expressed cell lineage appropriate phenotype markers
<i>Study purpose: bioengineered sphincters</i>				
Bioengineered Sphincters with smooth muscle and neural cells	Characterize the bioengineered sphincters	Bioengineered sphincters	Cadaver human and h-2kb-tsA58 immortomouse	<ul style="list-style-type: none">• Formation of intrinsically innervated sphincters• Bioengineered sphincters exhibited basal tone, relaxation and contractile activity
<i>Study purpose: implantation of bioengineered sphincters into rodent</i>				
Implantation of bioengineered sphincters	Optimization of the implantation procedure	Bioengineered sphincters	RAG1–/– mice	Bioengineered sphincters were implanted subcutaneously on athymic mice (RAG1–/–) model
<i>Study purpose: end points analysis</i>				
Bioengineered sphincters histopathology	Analysis of fibrosis/ inflammation and functional activity	Implanted bioengineered sphincters	RAG1–/– mice	<ul style="list-style-type: none">• No fibrosis or inflammation was observed in Bioengineered Sphincter implants• Harvested post-implant sphincters capable of maintaining basal tone, relaxation, and contractile activity

Table 2.
Summary of nonclinical study for safety and efficacy of bioengineered sphincters in athymic rodent model.

process took 9 days to generate an intrinsically innervated muscle constructs mimicking physiological functionality to native IAS tissues. The neural cell differentiation was further confirmed by positive expression of mature excitatory (choline acetyltransferase; ChAT) and inhibitory (VIP) motor neurons in the quantitative analysis using PCR. The cross-sections of engineered sphincters were demonstrated positive immunoreactivity against ChAT and VIP markers. After physiological functional analysis, the bioengineered sphincter were implanted subcutaneously into immune suppressed RAG1–/– mice for 4 weeks [35].

At harvest, the implanted construct exhibited neo-vascularization without any symptom of fibrosis or immunogenic reaction. The immuno-histological analysis confirmed that the sections of the harvested implant displayed reticulated

neural network innervated into intact aligned muscles. The section displayed microvasculature and several blood vessels embedded within the implanted smooth muscles [35].

The myogenic and neuronal components were preserved after implantation. All the bioengineered constructs were able to generate myogenic spontaneous basal tone pre- and post-implantation. A rapid and robust relaxation response was observed against VIP. This relaxation was 50–70% attenuated on pre-treatment of TTX, indicated that VIP-induced relaxation has both neuronal, as well as myogenic component. The relaxation was further validated with EFS and resulted in transient relaxation ultimately recovered to basal tone. The inhibition of nitrergic and VIP-ergic EFS-induced relaxation (by antagonizing nitric oxide synthesis or receptor interaction) confirmed the relaxation of enteric nerves results in nitrergic as well as VIP-ergic inhibitory neurotransmission in the implants. The excitatory neurotransmitter Ach (and partial inhibition on pre-treatment with TTX)-induced contraction response emulated before and after implantation, confirmed synergistic involvement of both neuronal and myogenic components. Fundamental electromechanical coupling of smooth muscle was also maintained during implantation, rendering the implanted IAS physiologically similar to in vivo IAS [35].

This was the first attempt of bioengineering of intrinsically innervated human IAS constructs. Both of myogenic and neuronal components of constructs were stable, sustained, viable and synergistically responsive after implantation in immune-suppressed mice. The study also concluded that bioengineering of intrinsically innervated sphincter is feasible, scalable, and customizable to match specific size and cell population. This leads to one step closer towards bioengineering of human engineered BioSphincters.

6. Bioengineering of physiologically functional intrinsically innervated human internal anal sphincter constructs

In previous studies, IAS smooth muscle constructs were engineered [34, 40] and implanted for cytocompatibility and physiological analysis. These preliminary studies were proof of concept using human origin SMCs and immortalized human origin neural stem cells. To translate the bioengineered sphincter to the clinical realm, it was essential to use human origin neural cells to engineer IAS sphincters.

The next objective was to develop bioengineering physiologically functional, intrinsically innervated human IAS tissues, using human origin neural cells and IAS muscle cells. Therefore, a method was optimized for the isolation of neuronal progenitor cells (NPCs) from intestinal biopsies of adult human donors. The cell culture and characterization protocol were standardized to yield an undifferentiated pure population of enteric neural progenitor cells [33].

Several matrix compositions were evaluated as a carrier for differentiation of adult enteric NPCs to functional neurons. The type-1 collagen with laminin was optimized as hydrogel for neural differentiation [41, 42]. The collagen acts as a matrix for mechanical strength and laminin is important for neuronal development. The SMCs has the ability to reform the collagen hydrogel into 3D structure due to matrix metalloproteinase activity [43]. During this restructure of hydrogel from 2D to 3D, SMCs came into close proximities of NPCs and enhanced the NPCs differentiation. Detail NPCs-SMCs interactions were studied, and it was observed that mature smooth muscle was essential for the direct differentiation of adult enteric NPCs [33]. The ratio of NPCs and SMCs were also studied and concluded that 200,000 NPCs/construct with 500,000 SMC/constructs were optimum do generate a native physiological response [33].

The constructs responded appropriately to physiologically relevant stimulatory and inhibitory neurotransmitters during functional analysis. It was validated in immunocytochemistry, the intrinsically innervated bioengineered construct exhibited excitatory and inhibitory motor neuronal population. The constructs displayed characteristics of functional mature contractile IAS smooth muscle as well. Overall, the human innervated functional IAS sphincter like tissues were successfully bioengineered and characterized [33].

7. Peri-anal implantation of bioengineered human internal anal sphincter constructs intrinsically innervated with human neural progenitor cells

After successful bioengineering of human IAS sphincter-like tissues, it was essential to evaluate the in vivo safety and functionality. In the next part of the study, a method was developed for isolation of rectal verge in an athymic rodent

Steps	Study objective (s)	Test article	Animal model	Key outcome (e.g., safety (tumor/tox/ biodistribution), efficacy, characterization, stability, degradation)
Study purpose: cell isolation				
Isolation of SMC	To isolate IAS smooth muscle cells (SMC) and characterization	In vitro expanded IAS smooth muscle cells	Cadaver human	Smooth muscle cells expressed cell lineage appropriate phenotype markers
Isolation of neural progenitor cells	To isolate neural progenitor cells and characterization	In vitro expanded neural progenitor cells	Cadaver human	Neural progenitor cells expressed cell lineage appropriate phenotype markers
Study purpose: bioengineered sphincters				
Bioengineered sphincters with smooth muscle and neural progenitor cells	Characterize the bioengineered sphincters	Bioengineered sphincters	Cadaver human	<ul style="list-style-type: none">• Formation of intrinsically innervated sphincters• Bioengineered sphincters exhibited basal tone, relaxation, and contractile activity
Study purpose: implantation of bioengineered sphincters into rodent				
Implantation of bioengineered sphincters	Optimization of the implantation procedure	Bioengineered sphincters	athymic nude rats	Bioengineered sphincters were implanted peri-anal site on athymic nude rats model
Study purpose: end points analysis				
Bioengineered sphincters histopathology	Analysis of fibrosis/ inflammation and functional activity	Implanted bioengineered sphincters	athymic nude rats	<ul style="list-style-type: none">• No fibrosis or inflammation was observed in Bioengineered Sphincter implants• Harvested post-implant sphincters capable of maintaining basal tone, relaxation, and contractile activity

Table 3.
Summary of nonclinical study of safety and efficacy of peri-anal implantation of human origin bioengineered sphincters into athymic rodent model.

model. Athymic nude mice were larger animal compared to normal mice. The selection of immune deficient rat for implantation studies of human-origin bioengineered constructs was to avoid any immune rejection.

The intrinsically innervated human IAS Sphincter were bioengineered using IAS origin SMCs and enteric NPCs. The developed surgical models were used to implant bioengineered sphincter into the perianal region of athymic rats for 4 weeks, following assessment of viability and functionality [36]. All the rats survived till respective time points without any obstruction or difficulty with defecation or fecal accumulation. Histopathology analysis concluded the absence of any abscess formations, infection, or adverse reaction. The implanted constructs were stable and intact at perirectal tissue of the rat, without any sign of fibrosis or neoplasia. Immuno-histological analysis with endothelial-specific antigen, von Willebrand's factor confirmed neovascularization and formation of several blood vessels. The contractile smooth muscle phenotype was maintained by exhibiting positive expression to human reactive muscle specific antibodies. **Table 3** summarized the detail study design [36].

Pre- and post-implant physiological force measurement studies confirmed distinct characteristics like native sphincters. The engineered IAS sphincter exhibited stable spontaneous myogenic basal tone. There was a robust response to different relaxant and excitatory stimulants, which was persistent after implantation.

This study concluded that for clinical application the bioengineered sphincter could be used in an additive manner rather than in a replacement manner, where native compromised IAS sphincter can be supported by transplantation of additional bioengineered sphincters. In this way, the patient's own IAS can be preserved and augmented with additional autologous functional neuro-muscular components [36].

8. Long-term non-clinical study of autologous bioengineered BioSphincters for the treatment of fecal incontinence

This study aimed to provide data for a large animal model in support of the use of Bioengineered sphincter as a new therapy to treat FI. These nonclinical studies were conducted to test the safety and efficacy of using autologous cell bioengineered sphincters as a regenerative medicine approach for treating induced FI in rabbits. The study design consisted of four steps. **Table 4** summarizes the four steps including their objectives and key outcomes.

8.1 Selection of a large animal model for nonclinical studies of fecal incontinence

Currently, there is no model for FI where the defect is specific to the internal anal sphincter. In humans, the IAS is responsible for 70% of anal basal pressure, anal closure, and fecal continence. The New Zealand white rabbit (female, 3.0–3.5 kg at the enrollment of the study) was chosen as an animal model because the anatomy and the surgical planes of the anal area are similar to humans. The rabbit was selected as a good model for successful identification and surgical resection of full thickness biopsies with a successful outcome. Thus, the rabbit is a good large animal model for our lab to utilize in evaluating FI. The number of animals, experimental protocols, and overall study design used in this study were reviewed and approved by the Wake Forest Institutional Animal Care and Use Committee before conducting any component of this study involving animals. Each rabbit was given a unique identification number that was printed on the cage card. Each rabbit was identified using a unique identification number. All data collected on each animal was referenced with the unique animal identification number and tattooed onto the

Steps	Study objective(s)	Test article	Animal model	Key outcome (e.g., safety (tumor/tox/ biodistribution), efficacy, characterization, stability, degradation)
<i>Study purpose: developing FI model and autologous cell isolation</i>				
IAS hemi-sphincterectomy	To induce FI	Donor IAS tissue	Female New Zealand rabbits	Lack of fecal hygiene and significant reduction in anal basal pressure and RAIR
Isolation of SMC	To isolate autologous IAS smooth muscle cells (SMC) characterization of autologous smooth muscle	In vitro expanded IAS smooth muscle cells	Female New Zealand rabbits	Smooth muscle cells expressed cell lineage appropriate phenotype markers
Small intestinal biopsy	To isolate neural progenitor cells (NPC) characterization of autologous NPC	In vitro expanded small intestine neural progenitor cells	female New Zealand rabbits	Neural progenitor cells expressed cell lineage appropriate phenotype markers
<i>Study purpose: autologous bioengineered sphincters</i>				
Bioengineered sphincters with autologous smooth muscle and neural progenitor cells	Characterize the bioengineered sphincters	Autologous bioengineered sphincters	female New Zealand rabbits	Restoration of fecal hygiene, anal basal pressure, and RAIR
<i>Study purpose: implantation of engineered autologous bioengineered sphincters to treat FI in rabbits</i>				
Implantation of bioengineered sphincters	Optimization of the implantation procedure	Autologous bioengineered sphincters	female New Zealand rabbits	The dosage of bioengineered sphincters was optimized four bioengineered sphincters were implanted on each rabbit in the treated group
Anal basal pressure and RAIR	Effects of bioengineered sphincters on the restoration of continence	Autologous bioengineered sphincters	female New Zealand rabbits	Rabbits with induced FI receiving bioengineered sphincter implants had anal basal pressure, and RAIR restored to normal baseline, but rabbits with induced FI in the non-treated group and sham surgery group had consistently reduced anal basal pressure and RAIR
<i>Study purpose: end points analysis</i>				
Blood results	Effects of implants on blood cell counts, kidney and liver function, and electrolytes.	Implanted bioengineered sphincters	female New Zealand rabbits	There were no adverse effects of implants on blood values
Tissue pathology	Effects of experimental conditions on tissue pathology	Implanted bioengineered sphincters	female New Zealand rabbits	There were no effects of experimental condition on local or peripheral histopathology

Steps	Study objective(s)	Test article	Animal model	Key outcome (e.g., safety (tumor/tox/ biodistribution), efficacy, characterization, stability, degradation)
Clinical presentation	Morbidity/mortality	Implanted bioengineered sphincters	female New Zealand rabbits	There were no effects of bioengineered sphincter implantation on morbidity or mortality
IAS histopathology	Fibrosis/inflammation	Implanted bioengineered sphincters bioengineered sphincter	female New Zealand rabbits	No definitive difference between bioengineered sphincter implants and naïve. No evidence of neoplasia

Table 4.
Summary of nonclinical study of safety and efficacy of bioengineered sphincters.

ear of each animal to prevent mix-up. Rabbits were acclimated for at least 6 days before enrollment in the study [10, 39].

8.2 Study groups

The groups of the study, summarized in **Table 5**, was developed to assess the post-implantation safety of bioengineered sphincters in rabbits at three-time points (3, 6, and 12 months). All animals underwent IAS hemi-sphincterectomy to induce FI. Rabbits were randomly divided into three experimental groups: (1) non-treated group (incontinent control), (2) treated group (received surgical implantation of bioengineered sphincters 6–8 weeks following sphincterectomy through a surgical opening of the anal verge), and (3) Sham surgery group (surgical opening of the anal verge was performed followed by immediate closure without implantation of bioengineered sphincters).

8.2.1 Development of FI

The IAS hemi-sphincterectomy was performed on all the rabbits to induce passive FI. The development of passive FI was confirmed in each assessment of fecal hygiene and anorectal pressure. Baseline manometry readings were obtained on all rabbits before any surgeries. Following hemi-sphincterectomy, anorectal manometry was performed on all rabbits to confirm passive FI, which was identified by lack of fecal hygiene and by a significant decrease in anal basal pressure and RAIR in all rabbits [10, 39].

8.2.2 Bioengineering of autologous BioSphincters

The SMCs were isolated from the IAS harvested during hemi-sphincterectomy. Isolated cells were characterized by α -smooth muscle actin and smoothelin markers. Cells stained positive confirming contractile phenotype of smooth muscle cells. NPCs were isolated from small intestine biopsies. Cells were then characterized by immunofluorescence and stained positive for p75NTR, Nestin, and Sox2, confirming neural crest-derived stem cells. Both cell types were expanded for 4 weeks to obtain the required number to form the bioengineered sphincters.

Intrinsically innervated IAS sphincters were bioengineered using both types of cells as described previously. Bioengineered sphincter products were characterized using different methods. The presence of aligned smooth muscle cells and the

Study groups (no. of rabbits)	Baseline manometry	Sphincterectomy to induce FI	Manometry post sphincterectomy	4–6 weeks post sphincterectomy	1 month 3 months 6 months 12 months
Non-treated group (11)	✓	✓	✓	No treatment	Manometry post sphincterectomy
Treated group (10)	✓	✓	✓	Implant bioengineered sphincters	Manometry post implant
Sham surgery group (5)	✓	✓	✓	Sham surgery	Manometry post sham

Table 5.
Study groups for the non-clinical study.

differentiated functional neural network was confirmed via immune-reactivity against smoothelin and β III tubulin. These results further validated via positive expression of smoothelin and β III tubulin qPCR. Engineered IAS sphincters were tested for physiological functionality. The engineered tissues able to generate the spontaneous basal tone and exhibited a robust stable response following pharmacological or electrical stimuli. The bioengineered autologous BioSphincters were implanted adjacent to IAS tissues into the respective rabbits [10, 39].

8.2.3 Implantation and restoration of fecal continence

Anorectal manometry is a technique used to measure contractility in the anus and rectum. Anorectal manometry was performed initially at baseline prior to any surgery. These measurements reflected the control state for all animals in this study. Anorectal manometry was performed prior to any surgery (before animals went for any procedure) to record the baseline, and 1 month following IAS hemi-sphincterectomy (biopsy), then at 3, 6, and 12 months in each experimental group.

8.2.3.1 Restoration of anorectal pressure

IAS hemi-sphincterectomy resulted in a significant decrease in anal basal pressure and RAIR compared to baseline (no surgery), supporting the validity of the induced-incontinence model. In the sham surgery group, anal basal pressure and RAIR were not improved and were comparable to readings from rabbits in the non-treated group. Compared to baseline, the basal pressure in non-treated and sham group was decreased by 41% ($p < 0.0001$) after 1 month of hemi-sphincterectomy and remained low up to study time point of 12 months. Similarly, RAIR was also reduced by 50.9% from the baseline ($p < 0.0001$). It remained low in non-treated group (49.2%) and sham groups (40.0%) compared to baseline till the study time point.

This reduced anorectal functionality was restored within 1-month post-implantation of autologous BioSphincters in the treated group. The resting pressure was returned to baseline after 4 weeks of implantation and remained similar up to 12 months. RAIR was restored by $\sim 88\%$ in initial 1 month and improved within 3 months and sustained till 12 months. The restoration of basal pressure and RAIR were significantly higher ($p < 0.0001$) than values observed in the non-treated group and sham groups.

8.2.3.2 Improvement in fecal hygiene

The IAS hemi-sphincterectomy affected fecal hygiene of the rabbits. This was evident from messy rabbit cages as feces were dispersed over the whole area of the cage. There was a definite lack of anal area hygiene as the area was always covered in a thin layer of feces. After implantation, the fecal hygiene returned to normal with a clean anal area and normal defecatory movement.

An improvement in defecatory activity was observed as early as 3 weeks after implantation of the bioengineered sphincters. Stool consistency returned to a firm pellet, similar to what was observed before FI was induced by the sphincterectomy.

8.2.3.3 Histopathology assessment

The post-implant harvested tissues displayed intact BioSphincter after 12 months of implantation. The presence of a thick continuous sheet of muscles innervated with neuronal network validated the manometry outcomes. There was the absence of any

Figure 2. Different stages from bioengineered sphincter to implantation; (A) bioengineered sphincter; (B) implantation of two bioengineered sphincters; (C) 4 implanted bioengineered sphincters; and (D) implanted bioengineered sphincter after euthanasia (after 12 months of implantation).

fibrosis or avascular collagen around the implant, indicating no foreign-body reaction with the implants. Pathologic findings in this study were generally minor and consisted primarily of a low incidence of background changes and minor changes attributable to implantation. There was no evidence of neoplasia. These results confirmed that the bioengineered sphincters were viable and functional in vivo with the maintenance of both the muscle and neural components [10, 39].

In this study, passive FI was successfully developed in the large animal model. The bioengineered intrinsically innervated IAS constructs from the autologous cells retrieved at biopsy. The IAS constructs were bioengineered and implanted after 6–8 weeks after harvesting the cells (**Figure 2A**); then, one by one, four bioengineered sphincters were implanted at the anal site (**Figure 2B**). The four bioengineered sphincters were stacked together at the site (**Figure 2C**). After 12 months of implantation, implanted bioengineered sphincters appeared intact as one tissue at the site (**Figure 2D**).

The animals resumed normal activity and defecatory bowel movement. There was no indication of any rectal outlet obstruction or anal stenosis. Anorectal manometry was performed on the animals monthly beginning 6 weeks after implantation. The animals exhibited a reinstated basal tone and RAIR. Animals were maintained and monitored up to 12 months after implantation. At each endpoint, after euthanasia, the harvested implant was tested. Results show that the construct maintained physiological functionality. The tests show that both muscle and neural type of cells maintained their physiological function. In other experiments, we have demonstrated that the cells of the implant stayed within the implant and did not migrate outside the location of the implant.

9. Conclusion

Regeneration of an intrinsically innervated function IAS sphincter is a promising approach for long-term relief from passive FI. The IAS muscle and neural cells synergized in collagen-laminin hydrogel as a 3D sphincter like architecture, mimicking the native IAS cell orientation and innervation. The bioengineering process has been optimized, scaled up for clinical application using human origin cells. The signaling pathways for sphincter tone and contraction were characterized. The bioengineered sphincter able to generate spontaneous tone and response to different pharmacological agents was comparable to human IAS. The stability, viability and cytocompatibility analysis of engineered sphincters were carried out in vitro and in vivo conditions. The step-wise pre-clinical assessment of engineered

autologous BioSphincters confirmed biocompatibility as IAS sphincter substitute, without any adverse effect. The implanted autologous BioSphincters vascularized, integrated with the impaired native IAS and regenerated stable, circularly oriented IAS muscle population, innervated with the neural network. The regeneration approach provided immediate symptomatic relief by restoration fecal hygiene. We have developed a large animal model of passive fecal incontinence and demonstrated sustained restoration of fecal continence, and restoration of basal tone and restoration of RAIR in this model after implantation of engineered autologous intrinsically innervated internal anal sphincter (IAS) BioSphincters. In a clinical scenario, this innovative approach will be able to reinstate continence, by providing an additive functional intrinsically innervated IAS bioengineered from the patient's cells.

As summary, regeneration, and implantation of the IAS BioSphincter will benefit a large socially distressed segment of the population via restoration of physiological function of the IAS, resolve FI, and improving quality of life.

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Conflict of interest

KNB is the founder of CELLF BIO LLC a startup biotech that has an interest in developing treatments for neurodegenerative diseases of the gut.

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References

- [1] Rao SS. Pathophysiology of adult fecal incontinence. *Gastroenterology*. 2004;**126**(1 Suppl 1):S14-S22. DOI: 10.1053/j.gastro.2003.10.013
- [2] SSCC R, Bharucha AE, Chiarioni G, et al. Anorectal disorders. *Gastroenterology*. 2018;**150**(6): 1430-1442.e4. DOI: 10.1053/j.gastro.2016.02.009
- [3] Bartlett L, Nowak M, Ho Y-H. Impact of fecal incontinence on quality of life. *World Journal of Gastroenterology*. 2009;**15**(26): 3276-3282
- [4] Lazarescu A, Turnbull GK, Vanner S. Investigating and treating fecal incontinence: When and how. *Canadian Journal of Gastroenterology*. 2009; **23**(4):301-308. Available from: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2711681/>
- [5] Goode PS, Burgio KL, Halli AD, et al. Prevalence and correlates of fecal incontinence in community-dwelling older adults. *Journal of the American Geriatrics Society*. 2005;**53**(4): 629-635. DOI: 10.1111/j.1532-5415.2005.53211.x
- [6] Engel AF, Kamm MA, Bartram CI, Nicholls RJ. Relationship of symptoms in faecal incontinence to specific sphincter abnormalities. *International Journal of Colorectal Disease*. 1995; **10**(3):152-155. DOI: 10.1007/BF00298538
- [7] Bharucha AE. Pelvic floor: Anatomy and function. *Neurogastroenterology and Motility*. 2006;**18**(7):507-519. DOI: 10.1111/j.1365-2982.2006.00803.x
- [8] Rattan S. The internal anal sphincter: Regulation of smooth muscle tone and relaxation. *Neurogastroenterology and Motility*. 2005;**17**(Suppl 1):50-59. DOI: 10.1111/j.1365-2982.2005.00659.x
- [9] Bharucha AEA, Dunivan G, Goode PPS, et al. Epidemiology, pathophysiology, and classification of fecal incontinence: State of the science summary for the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) workshop. *The American Journal of Gastroenterology*. 2015;**110**(1):127-136. DOI: 10.1038/ajg.2014.396
- [10] Bohl JL, Zakhem E, Bitar KN, Autonomic I, Neurological N. Successful treatment of passive Fecal incontinence in an animal model using engineered Biosphincters: A 3 month follow-up study. *Stem Cells Translational Medicine*. 2017;**6**(9):1795-1802. DOI: 10.1002/sctm.16-0458
- [11] Huebner M, Margulies RU, Fenner DE, et al. Age effects on internal anal sphincter thickness and diameter in nulliparous females. *Diseases of the Colon and Rectum*. 2007;**50**(9):1405-1411. DOI: 10.1007/s10350-006-0877-7
- [12] Lindsey I, Jones OM, Smilgin-Humphreys MM, Cunningham C, Mortensen NJ. Patterns of fecal incontinence after anal surgery. *Diseases of the Colon and Rectum*. 2004;**47**(10):1643-1649
- [13] Whitehead WE, Rao SSC, Lowry A, et al. Treatment of fecal incontinence: State of the science summary for the National Institute of Diabetes and Digestive and Kidney Diseases workshop. *The American Journal of Gastroenterology*. 2015;**110**(1):138-146. DOI: 10.1038/ajg.2014.303
- [14] Bliss DZ, Jung HJ, Savik K, et al. Supplementation with dietary fiber improves fecal incontinence. *Nursing Research*. 2001;**50**(4):203-213. DOI: 10.1097/00006199-200107000-00004
- [15] Omar MI, Alexander CE. Drug treatment for faecal incontinence in

- adults. Cochrane Database of Systematic Reviews. 2013;(6):1-61. DOI: 10.1002/14651858.CD002116.pub2
- [16] Heymen S, Scarlett Y, Jones K, Ringel Y, Drossman D, Whitehead WE. Randomized controlled trial shows biofeedback to be superior to pelvic floor exercises for fecal incontinence. *Diseases of the Colon and Rectum*. 2009;**52**(10):1730-1737. DOI: 10.1007/DCR.0b013e3181b55455
- [17] Norton C, Chelvanayagam S, Wilson-Barnett J, et al. Randomized controlled trial of biofeedback for fecal incontinence. *Gastroenterology*. 2003; **125**(5):1320-1329. DOI: 10.1053/S0016-5085(03)01368-4
- [18] Leroi AM, Siproudhis L, Etienney I, et al. Transcutaneous electrical tibial nerve stimulation in the treatment of fecal incontinence: A randomized trial (CONSORT 1a). *The American Journal of Gastroenterology*. 2012; **107**(12):1888-1896. DOI: 10.1038/ajg.2012.330
- [19] Tan E, Ngo N-T, Darzi A, Shenouda M, Tekkis PP. Meta-analysis: Sacral nerve stimulation versus conservative therapy in the treatment of faecal incontinence. *International Journal of Colorectal Disease*. 2011;**26**(3):275-294. DOI: 10.1007/s00384-010-1119-y
- [20] Hull T, Giese C, Wexner SD, et al. Long-term durability of sacral nerve stimulation therapy for chronic fecal incontinence. *Diseases of the Colon and Rectum*. 2013;**56**(2):234-245. DOI: 10.1097/DCR.0b013e318276b24c
- [21] Wong MTC, Meurette G, Wyart V, Glemain P, Lehur P-A. The artificial bowel sphincter: A single institution experience over a decade. *Annals of Surgery*. 2011;**254**(6):951-956. DOI: 10.1097/SLA.0b013e31823ac2bc
- [22] Lehur P-A, McNevin S, Buntzen S, Mellgren AF, Laurberg S, Madoff RD. Magnetic anal sphincter augmentation for the treatment of fecal incontinence: A preliminary report from a feasibility study. *Diseases of the Colon and Rectum*. 2010;**53**(12):1604-1610. DOI: 10.1007/DCR.0b013e3181f5d5f7
- [23] Luo C, Samaranayake CB, Plank LD, Bissett IP. Systematic review on the efficacy and safety of injectable bulking agents for passive faecal incontinence. *Colorectal Disease*. 2010;**12**(4):296-303. DOI: 10.1111/j.1463-1318.2009.01828.x
- [24] Mellgren A, Matzel KE, Pollack J, Hull T, Bernstein M, Graf W. Long-term efficacy of NASHA Dx injection therapy for treatment of fecal incontinence. *Neurogastroenterology and Motility*. 2014;**26**(8):1087-1094. DOI: 10.1111/nmo.12360
- [25] Singh J, Rattan S. Bioengineered human IAS reconstructs with functional and molecular properties similar to intact IAS. *American Journal of Physiology. Gastrointestinal and Liver Physiology*. 2012;**303**(6):G713-G722. DOI: 10.1152/ajpgi.00112.2012
- [26] Kajbafzadeh A-M, Elmi A, Talab SS, Esfahani SA, Tourchi A. Functional external anal sphincter reconstruction for treatment of anal incontinence using muscle progenitor cell auto grafting. *Diseases of the Colon and Rectum*. 2010; **53**(10):1415-1421. DOI: 10.1007/DCR.0b013e3181e53088
- [27] Sarveazad A, Newstead GL, Mirzaei R, et al. A new method for treating fecal incontinence by implanting stem cells derived from human adipose tissue: Preliminary findings of a randomized double-blind clinical trial. *Stem Cell Research & Therapy*. 2017;**8**(1):40. DOI: 10.1186/s13287-017-0489-2
- [28] Frudinger A, Kolle D, Schwaiger W, Pfeifer J, Paede J, Halligan S. Muscle-derived cell injection to treat anal incontinence due to obstetric trauma: Pilot study with 1 follow-up.

Gut. 2010;**59**(1):55-61. DOI: 10.1136/gut.2009.181347

[29] Tan JJY, Chan M, Tjandra JJ. Evolving therapy for fecal incontinence. *Diseases of the Colon and Rectum*. 2007; **50**(11):1950-1967. DOI: 10.1007/s10350-007-9009-2

[30] Pathi SD, Acevedo JF, Keller PW, et al. Recovery of the injured external anal sphincter after injection of local or intravenous mesenchymal stem cells. *Obstetrics and Gynecology*. 2012;**119**(1): 134-144. DOI: 10.1097/AOG.0b013e3182397009

[31] Jorge MJN, Wexner SD. Etiology and management of fecal incontinence. *Diseases of the Colon and Rectum*. 1993;**36**(1):77-97. DOI: 10.1007/BF02050307

[32] White AB, Keller PW, Acevedo JF, Word RA, Wai CY. Effect of myogenic stem cells on contractile properties of the repaired and unrepaired transected external anal sphincter in an animal model. *Obstetrics and Gynecology*. 2010;**115**(4):815-823. DOI: 10.1097/AOG.0b013e3181d56cc5

[33] Gilmont RR, Raghavan S, Somara S, Bitar KN. Bioengineering of physiologically functional intrinsically innervated human internal anal sphincter constructs. *Tissue Engineering. Part A*. 2014;**20**(11-12): 1603-1611. DOI: 10.1089/ten.tea.2013.0422

[34] Somara S, Gilmont RR, Dennis RG, Bitar KN. Bioengineered internal anal sphincter derived from isolated human internal anal sphincter smooth muscle cells. *Gastroenterology*. 2009;**137**(1): 53-61. DOI: 10.1053/j.gastro.2009.03.036

[35] Raghavan S, Gilmont RR, Miyasaka EA, et al. Successful implantation of bioengineered, intrinsically innervated, human internal anal sphincter.

Gastroenterology. 2011;**141**(1):310-319. DOI: 10.1053/j.gastro.2011.03.056

[36] Raghavan S, Miyasaka EA, Gilmont RR, Somara S, Teitelbaum DH, Bitar KN. Perianal implantation of bioengineered human internal anal sphincter constructs intrinsically innervated with human neural progenitor cells. *Surgery*. 2014;**155**(4): 668-674. DOI: 10.1016/j.surg.2013.12.023

[37] Hashish M, Raghavan S, Somara S, et al. Surgical implantation of a bioengineered internal anal sphincter. *Journal of Pediatric Surgery*. 2010; **45**(1):52-58. DOI: 10.1016/j.jpedsurg.2009.10.010

[38] Raghavan S, Miyasaka EA, Hashish M, et al. Successful implantation of physiologically functional bioengineered mouse internal anal sphincter. *American Journal of Physiology. Gastrointestinal and Liver Physiology*. 2010;**299**(2):G430-G439. DOI: 10.1152/ajpgi.00269.2009

[39] Zakhem E, Bohl JL, Tamburrini R, et al. Therapeutic potential of lab-grown autologous BioSphincters for the treatment of fecal incontinence in a long-term pre-clinical study. *Advanced Research in Gastroenterology & Hepatology*. 2019;**12**(4):555845

[40] Hecker L, Baar K, Dennis RGRRG, Bitar KN. Development of a three-dimensional physiological model of the internal anal sphincter bioengineered in vitro from isolated smooth muscle cells. *American Journal of Physiology. Gastrointestinal and Liver Physiology*. 2005;**0656**(2):188-196. DOI: 10.1152/ajpgi.00335.2004

[41] Rego SL, Raghavan S, Zakhem E, Bitar KN. Enteric neural differentiation in innervated, physiologically functional, smooth muscle constructs is modulated by bone morphogenic protein 2 secreted by sphincteric smooth

muscle cells. *Journal of Tissue Engineering and Regenerative Medicine*. 2017;**11**(4):1251-1261. DOI: 10.1002/term.2027

[42] Raghavan S, Bitar KN. The influence of extracellular matrix composition on the differentiation of neuronal subtypes in tissue engineered innervated intestinal smooth muscle sheets. *Biomaterials*. 2014;**35**(26):7429-7440. DOI: 10.1016/j.biomaterials.2014.05.037

[43] Ceresa CC, Knox AJ, Johnson SR. Use of a three-dimensional cell culture model to study airway smooth muscle-mast cell interactions in airway remodeling. *American Journal of Physiology. Lung Cellular and Molecular Physiology*. 2009;**296**(6):L1059-L1066. DOI: 10.1152/ajplung.90445.2008