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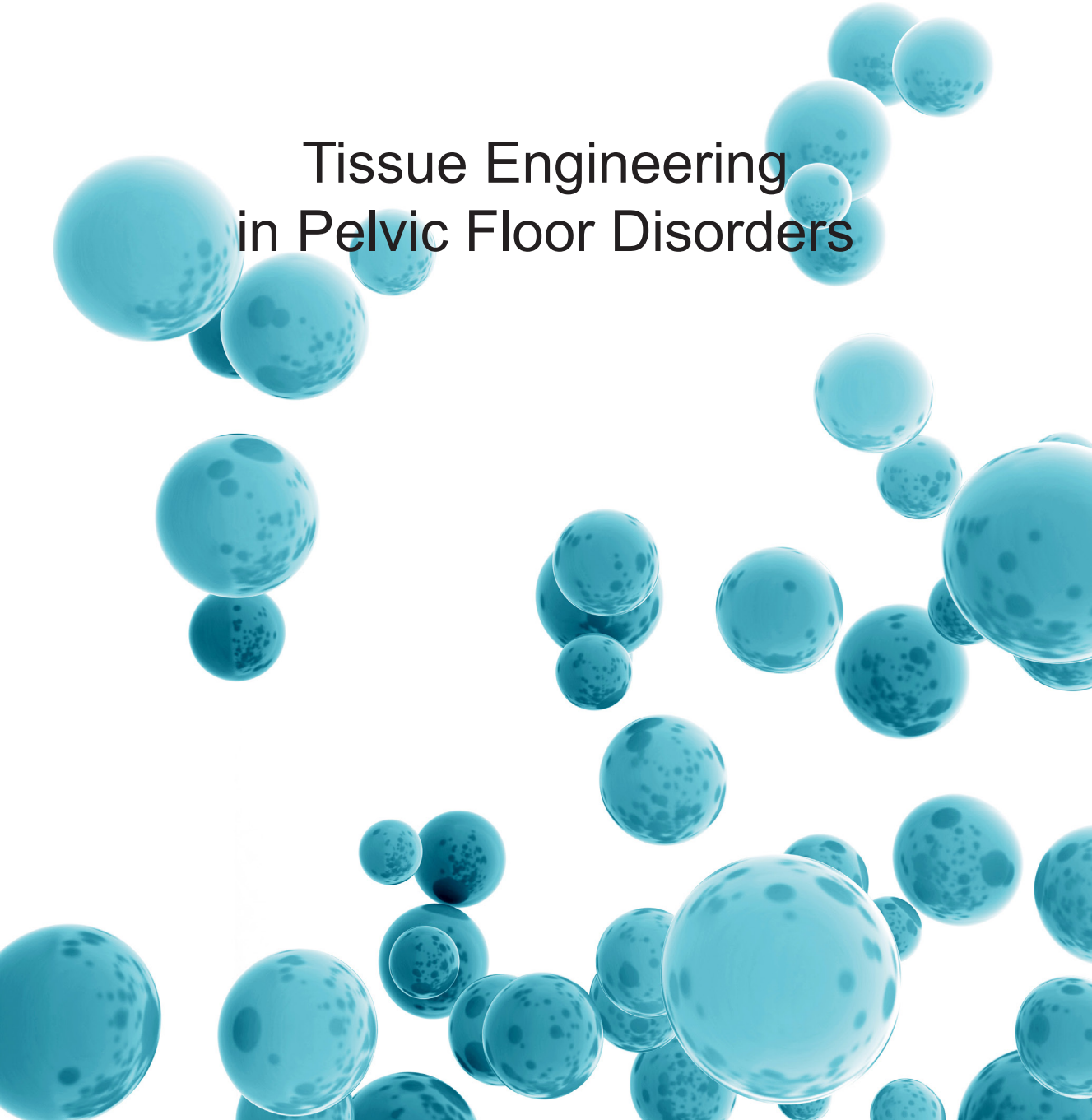
Tissue Engineering in Pelvic Floor Disorders



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Tissue Engineering in Pelvic Floor Disorders

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ABSTRACT

Anal incontinence (AI), urinary incontinence (UI) and pelvic organ prolapse (POP) are common conditions and surgical interventions are often required for their successful treatment. Other conditions such as vaginal epithelial deficiencies after pelvic surgery, congenital vaginal agenesis or transgender genital reconstructions are relatively rare conditions that frequently necessitate advanced surgical techniques and materials for their treatment. Our aim was to assess possibilities of tissue engineering as a method for treating pelvic floor disorders.

In Study I we analyzed the AI symptoms of 322 women who suffered a third or fourth degree obstetric anal sphincter injury during 2007-2013 in Tampere University Hospital. The aim was to identify risk factors for persisting symptoms of AI at six months after primary repair of the anal sphincter, to evaluate the usefulness of a simple three-choice assessment form combined with the Wexner Incontinence Score (WIS) and to evaluate the need for alternative treatment methods. According to the three-choice assessment, 63% of the women had no AI symptoms, 27% had mild symptoms and 9% had severe AI symptoms and wanted further treatment. Risk factors for AI symptoms were instrumental vaginal delivery, more severe types of injury and advancing maternal age. There was a good correlation between the three-choice assessment and WIS. Thus, the three-choice assessment might be a useful tool to take the woman's own wish into consideration to better target health care resources when assessing the need for further treatment.

In Study II our aim was to develop a tissue engineering method for acute anal sphincter rupture and thus help to create a novel treatment method for AI. Human adipose stem cells (hASCs) were combined with either polyacrylamide hydrogel (Bulkamid®) or saline combined with surgery to treat iatrogenic anal sphincter rupture in a rat model. The anorectal manometry revealed that both Bulkamid® and saline combined with hASCs produced better functional results than the controls without the hASCs. Bulkamid® was well integrated in the tissue and produced less inflammation than the hASC+saline-injections. There was no difference in muscle continuity between the groups according to the histological

and micro-computed tomography analysis which suggests that the effect of the hASCs was paracrine stimulation rather than direct sphincter muscle differentiation.

In a pilot study (Study III) adipose stem cell (ASC) injections were tested in a clinical trial. Five women suffering from stress or mixed urinary incontinence (SUI, MUI) were treated by transurethral autologous ASC-injections. The ASCs were collected from the patients' abdominal fat under local anesthesia and then processed and expanded in laboratory for three weeks before combining with collagen gel (Contigen®). The primary outcome measure was the cough test, secondary measures the urethrocystometry, 24h pad test and the subjective quality of life (QoL) assessments. As a result, three patients had a negative cough test and two out of five women gained reasonable continence and were satisfied with the treatment at one year. However, three patients were still incontinent and wished for an operation with mid-urethral tape. No serious side effects were observed.

In Study IV a new scaffold material, supercritical carbon dioxide (scCO2) foamed poly-l-lactide-ε-co-caprolactone (scPLCL) combined with vaginal epithelial and stromal cells was used to develop a reconstructive graft for treatment of epithelial defects, e.g. congenital vaginal agenesis. Vaginal epithelium was acquired from three transgender patients in elective vaginectomy operations. The vaginal epithelial and stromal cells were separated and expanded in culture and seeded on three-dimentional (3D) scPLCL scaffold specimens. The survival and phenotype of the cells were assessed at day 7 and 14. Both cell types survived well, and especially the epithelial cells were spread throughout the scaffolds favoring the pores of the scPLCL-scaffold. The tensile and elastic properties of the scPLCL-scaffold seemed adequate for vaginal tissue engineering.

Based on this thesis, we can conclude that new treatment methods for pelvic floor disorders are needed, especially for AI and recurrent UI, and tissue engineering based strategies are promising options for their treatment. However, more studies are needed to assure the safety and efficacy of these novel treatments.

TIIVISTELMÄ

Ulosteen- ja virtsankarkailu sekä synnytinelinten laskeuma ovat yleisiä ongelmia, jotka vaativat usein kirurgisia hoitomenetelmiä. Emättimen alueen limakalvopuutokset lantion alueen kirurgian jälkeen, synnynnäiset epämuodostumat ja transsukupuolisten sukuelinkirurgia ovat harvinaisia, mutta niiden kirurginen korjaaminen vaatii usein edistynyttä kirurgista tekniikkaa ja biomateriaaleja. Tutkimustemme tavoitteena oli arvioida uusia kudosteknologisia mahdollisuuksia lantionpohjan toimintahäiriöiden hoidossa.

Osatyössä I selvitimme Tampereen yliopistollisessa sairaalassa vuosina 2007–2013 synnyttäneiden 322 synnytysrepeämäpotilaan (3.- tai 4. asteen peräaukon sulkijalihaksen repeämä) ulosteenkarkailuoireita kuuden kuukauden kuluttua repeämän korjauksesta. Tutkimuksen tavoitteena oli tunnistaa ulosteenkarkailun riskitekijöitä, arvioida kolmen vaihtoehdon kyselykaavakkeen käytettävyyttä yhdistettynä Wexner-pisteytykseen oireiden kartoituksessa ja arvioida uusien hoitomenetelmien tarvetta. Tutkimuksemme perusteella 63 % naisista oli ulosteenkarkailun suhteen oireettomia, 27 %:lla oli lieviä oireita ja 9 %:lla oli vaikea ulosteenkarkailu, johon potilaat halusivat jatkohoitoa. Ulosteenkarkailun riskitekijöitä olivat instrumenttiavusteinen alatiesynnytys, sulkijalihasrepeämän vaikeusaste ja äidin ikä. Kolmen vaihtoehdon kysely ja Wexner-pisteytys korreloivat keskenään hyvin, joten kolmen vaihtoehdon kysely voisi olla hyödyllinen apuväline terveydenhuollon resurssien oikeassa kohdentamisessa selvitettäessä naisen omaa toivetta ulosteenkarkailun jatkohoidosta.

Osatyön II tavoitteena oli kehittää peräaukon sulkijalihasvaurioon ja täten ulosteenkarkailuun uutta kudosteknologista hoitoa. Tutkimuksessa ihmisestä eristetyt rasvan kantasolut yhdistettiin joko 0,9 % keittosuolaan tai polyakryyliamidigeeliin (Bulkamid®), ja pistoksilla hoidettiin kirurgisesti aiheutettua ja korjattua rotan peräaukon sulkijalihasvauriota. Peräaukon sulkijalihaspainetutkimuksen perusteella kantasoluilla hoidetuilla rotilla peräaukon sulkijalihaksen supistusvoima oli parempi kuin pelkkää keittosuolaa tai Bulkamid®-geeliä saaneilla kontrolleilla. Bulkamid® sulautui ympäröivään kudokseen hyvin ja

aiheutti vähemmän tulehdusreaktiota kuin kantasolut keittosuolaliuoksessa. Mikroskooppitutkimuksen ja mikro-tietokonetomografian mukaan peräaukon sulkijalihaksen jatkuvuus ei eronnut ryhmien välillä toisistaan, jonka perusteella voisi päätellä, että kantasoluvaikutus perustuu enemmän solujen väliseen vuorovaikutukseen kuin solujen varsinaiseen erilaistumiseen lihaskudokseksi.

Osatyö III oli kliininen pilottitutkimus, jossa naisten ponnistusvirtsankarkailua hoidettiin virtsaputken kautta annetuilla kantasolupistoksilla. Rasvan kantasolut kerättiin potilaan vatsan alueen ihonalaisrasvasta paikallispuudutuksessa ja viljeltiin laboratoriossa kolmen viikon ajan ennen yhdistämistä kollageenigeeliin (Contigen®). Tutkimuksen ensisijainen päätemuuttuja oli yskäisytesti, toissijaisina päätemuuttujina olivat uretrokystometria, 24 tunnin vaippatesti ja virtsankarkailun kyselytutkimukset. Hoidon tuloksena kolmen potilaan yskäisytesti oli vuoden seurannassa negatiivinen ja potilaista kaksi oli tyytyväisiä virtsankarkailun vähenemiseen. Kolme potilasta kärsi kuitenkin edelleen virtsankarkailusta ja he halusivat operatiivista hoitoa. Pistoshoidoista ei ollut vakavia haittavaikutuksia. Virtsankarkailun hoito kantasolu-kollageenipistoksilla oli tutkimuksen perusteella turvallinen.

Neljännessä osatyössä selvitettiin uudella hiilidioksidimenetelmällä valmistetun PLCL-biomateriaalin ja emättimen pinta- ja tukisolujen yhdistelmän soveltuvuutta emättimen limakalvopuutosten kudosteknologiseen hoitoon. Hoito voisi soveltua esimerkiksi synnynnäisen emättimen puuttumisen kirurgiseen hoitoon. Limakalvonäytteitä kerättiin kolmelta transsukupuoliselta potilaalta emättimenpoistoleikkauksen yhteydessä. Emättimen epiteeli- ja tukikudossolut eroteltiin ja viljeltiin, jonka jälkeen solut siirrettiin kasvamaan kolmiulotteiselle PLCL-materiaalille. Solujen kasvua ja fenotyyppiä seurattiin laboratoriomenetelmin viikon ja kahden viikon aikapisteissä. Molemmat solutyypit kasvoivat biomateriaalilla hyvin, erityisesti pintasolut suosivat PLCL-materiaalin huokosia. PLCL-biomateriaalin fysikaaliset ominaisuudet näyttäisivät sopivan hyvin emättimen alueen kudosteknologiseen hoitoon.

Tutkimustemme perusteella uusia hoitokeinoja lantionpohjan toimintahäiriöiden, erityisesti ulosteenkarkailun ja uusiutuneen virtsankarkailun hoitoon kaivataan, ja kudosteknologia tarjoaa varteenotettavia vaihtoehtoja uusiksi hoitomenetelmiksi. Lisätutkimuksia hoitojen turvallisuuden ja tehokkuuden selvittämiseksi kuitenkin tarvitaan.

LIST OF ABBREVIATIONS

AI	Anal incontinence
ADSC	Adipose-derived stem cells
ANOVA	Analysis of variance
ARM	anorectal manometry
ASC	Adipose stem cell
BMI	Body mass index
DNA	Deoxyribonucleic acid
BMSC	Bone-marrow stem cell
CD	Cluster of differentiation
CI	Confidence interval
DIS	Detrusor Instability Score
EAS	External anal sphincter
EAUS	Endoanal ultrasonography
ECM	Extracellular matrix
EMG	Electromyography
FI	Fecal incontinence

GFP	Green fluorescent protein
GMP	Good Manufacturing Practice
hASC	Human adipose stem cell
hCBSC	Human cord blood stem cells
H&E	Hematoxylin and Eosin
IAS	Internal anal sphincter
ISD	Intrinsic sphincter deficiency
IIQ-7	Incontinence Impact Questionnaire-short form
L/D	Live/dead
LPP	Leak point pressure
MDSC	Muscle derived stem cell
MRI	Magnetic resonance imaging
MRKH	Mayer-Rokitansky-Küster-Hauser
MSC	Mesenchymal stem cell
MUCP	Maximal urethral closure pressure
MUI	Mixed urinary incontinence
NaCl	Sodium chloride
OASI	Obstetric anal sphincter injury
OR	Odds ratio

PBS	Phosphate-buffered saline/solution
PGA	Polyglycolic acid
PLCL	Poly-l-lactide-co-ε-caprolactone
PLGA	poly-(lactide-*co*-glycolide)
POP	Pelvic organ prolapse
QoL	Quality of life
scCO2	Supercritical carbon dioxide
scPLCL	Supercritical carbon dioxide foamed poly-l-lactide-co-ε-caprolactone
SEM	Scanning electron microscopy
SMA	Smooth muscle actin
SNS	Sacral nerve stimulation
SNM	Sacral neuromodulation
SUI	Stress urinary incontinence
SVF	Stromal vascular fraction
UCM	Uretrocystometry
UDI-6	Urogenital Distress Inventory –short form
UI	Urinary incontinence
UISS	Urinary Incontinence Severity Score

UUI	Urge urinary incontinence
VAS	Visual Analog Scale
VEGF	Vascular endothelial growth factor
WIS	Wexner Incontinence Score
μCT	Micro-computed tomography
3D	Three-dimensional

LIST OF ORIGINAL PUBLICATIONS

I. Kuismanen K, Nieminen K, Karjalainen K, Lehto K, Uotila J. Outcomes of primary anal sphincter repair after obstetric injury and evaluation of a novel three-choice symptom assessment.Tech Coloproctol. 2018 Mar;22:209-214. httpr://doi.org/10.1007/210151-018-1770-9

II. Kuismanen K, Juntunen M Tuominen H, Narra Girish N, Huhtala H, Nieminen K, Hyttinen J, Miettinen S. Functional outcome of human adipose stem cell injections in rat anal sphincter acute injury model. Stem Cells Transl. Med. 2018 Jan;7:295-304. httpr://doi: 10.1002/sctm.17-0208

III. Kuismanen K, Sartoneva R, Haimi S, Mannerstrom B, Tomás E, Miettinen S, Nieminen K. Autologous Adipose stem cells in Treatment of Female Stress Urinary Incontinence: Results of a Pilot Study. Stem Cells Transl. Med. 2014 Aug;3(8):936-41. doi: 10.5966/sctm.2013-0197

IV. Sartoneva R, Kuismanen K, Juntunen M, Karjalainen S, Hannula M, Hyttinen J, Huhtala H, Paakinaho K, Miettinen S. Novel porous poly-l-lactide-ε-co-caprolactone (PLCL) scaffold is a potential biomaterial for vaginal epithelial tissue engineering. (Submitted in Royal Society Open Science)

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1 INTRODUCTION

Pelvic floor disorders include, e.g. anal incontinence (AI), urinary incontinence (UI), urinary storage disorders and pelvic organ prolapse (POP) (Haylen et al. 2010b; Bo et al. 2017). Even though they are not usually life threatening, pelvic floor disorders can have a substantial impact on the quality of life (QoL) (Lim et al. 2018; Jelovsek and Barber 2006; Brown et al. 2012). There has been development in conventional treatment methods of these disorders during past decades; however, there is a need for novel approaches. Additionally, serious operative complications in pelvic floor surgery and congenital disorders, e.g. vaginal agenesis, might need reconstruction with either synthetic or biological material (Chapple et al. 2015).

There are different estimates of the incidence of AI varying from 3% to 40% in the adult population, depending on the definition (Varma et al. 2006; Jerez-Roig et al. 2015). The most common cause of AI in women is obstetric trauma (Hayden and Weiss 2011). Recognition of the obstetric anal sphincter injury (OASI) is essential for adequate primary repair. Rehabilitation of the pelvic floor with biofeedback physiotherapy after delivery and especially after OASI is widely recommended (Fynes et al. 1999). Different questionnaires have been developed to detect patients with persistent AI symptoms. Primary repair is relatively successful since about 90% of women have no symptoms in short term (Ramalingam and Monga 2013). Studies about long term results are however heterogeneous and there are several different outcome measures used for detection of AI which makes the comparison of the treatment methods and studies challenging. Nowadays the most common treatment method for persistent AI is sacral neuromodulation (SNM), which, however, is an invasive and expensive surgical method.

The most common type of UI is stress urinary incontinence (SUI). The lifetime risk of undergoing surgery for SUI among women in developed countries varies between 6.6% and 13.9% (Wu et al. 2014; Kurkijärvi et al. 2016). A plethora of surgical techniques has been introduced to treat female SUI. Mid-urethral slings have become first-line surgical treatments for SUI because they are minimally

invasive, have excellent short term and good long term success rates, and require a relatively short learning curve (Virkud 2011). Other treatment methods such as bulking agents are an option especially for patients who are unresponsive to or not suitable for surgical treatment. However, injections with bulking agents have not been as effective as surgical methods, and the main problem has been the relatively poor sustainability of the bulking effect (Kirchin et al. 2012).

Other disorders that might need surgical intervention and novel surgical techniques utilizing new biomaterials are postoperative vaginal defects after surgery for POP or gynecological cancer, transgender genital reconstruction of vagina or urethra and congenital malformations, e.g. vaginal agenesis (Bako and Dhar 2009; Bacalbasa and Balescu 2015; Selvaggi and Bellringer 2011; Roberts et al. 2001).

Tissue engineering is a fairly juvenile branch of science that utilizes the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function. The aim is to develop novel methods to replace damaged tissues and maintain and enhance the functional properties of organs (Langer and Vacanti 1993). Regeneration in tissue engineering requires cells, signaling molecules and biomaterial as a carrier, e.g. scaffold or hydrogel.

Stem cells are considered the cell source with the most potential for tissue engineering. They are undifferentiated cells that are capable of proliferating indefinitely and able to differentiate into several pathways during the entire life of an organism. The traditional way of classifying stem cells is according to their differentiation potential: toti-, pluri- or multipotent cells (Choumerianou et al. 2008). Stem cells can also be classified into embryonic and adult stem cells (Lo Furno et al. 2016). Adult stem cells are further classified according to the cell source. Muscle derived stem cells (MDSCs) are the most widely-used stem cells in incontinence therapy and pelvic floor disorders (Boennelycke et al. 2013). Adipose stem cells (ASCs) are an ideal source for tissue engineering because they are easily accessible, have low immunogenity and the retrieval of tissue does not disturb the function (Gimble et al. 2007).

Biomaterials are fundamental elements of tissue engineering. They can be described as biologic materials of either natural or synthetic origin that are used for medical applications (Williams 2009). Biodegradable biomaterials are metabolized

by the biological environment; the degradation rate depends on the composition of the material as well as the individual properties of the microenvironment of the transplanted organ and patient. Foreign body reaction, inflammation and toxicity of a biocompatible biomaterial are low (Langer and Tirrell 2004). Biomaterials include natural polymers such as collagen, biological acellular tissue matrices such as dermis, or synthetic polymers such as poly (-ε)-esters (Nair and Laurencin 2007). Poly-l-lactide-ε-co-caprolactone (PLCL) scaffold is a novel synthetic biomaterial that has not previously been tested for vaginal tissue engineering.

In our studies our aim was to investigated the prevalence of AI symptoms after OASI primary repair and the need for novel treatment methods for AI in an obstetric population (Study I); the technique, efficiency and safety of ASC injection treatment in an AI simulated animal model (Study II); the clinical implementation of ASC injection in SUI treatment (Study III) and the usability of supercritical carbon dioxide foamed PLCL (scPLCL) scaffold in vaginal tissue engineering (Study IV). To the best of our knowledge, these are the first studies to test polyacrylamide hydrogel Bulkamid® and scPLCL-scaffold as biomaterials for tissue engineering translations in the treatment of female pelvic floor disorders.

2 REVIEW OF THE LITERATURE

2.1 Pelvic floor disorders

The pelvic floor or the pelvic diaphragm is a complex composition of pelvic floor muscles and connective tissue. The pelvic organs are lower urinary tract organs (bladder and urethra), genital organs (vulva, vagina, uterus, ovaries and fallopian tubes) and colorectal organs (anus, rectum, and colon). The bladder and rectum serve as a reservoir of bodily waste products (urine, feces) and the urethra and the anal sphincter are controlled by a complex system involving both autonomous and somatic nervous systems. Pelvic floor muscles (e.g. levator muscles, puborectalis muscle) have an important role in supporting the continence in exertion, e.g. coughing, straining and laughing (Patel and Chapple 2008b; Gurjar and Jones 2011) (Figure 1).

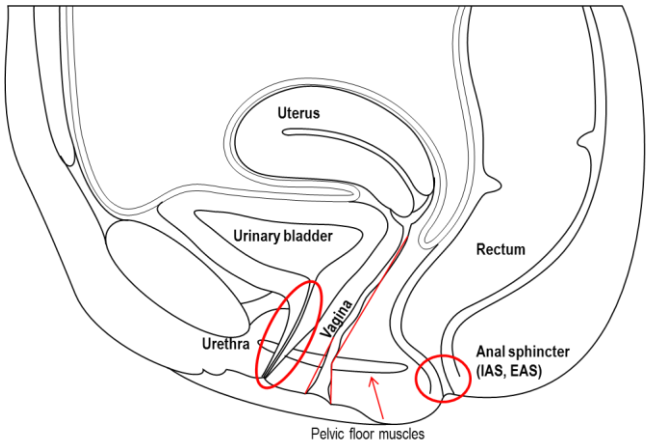

Figure 1. A schematic drawing of the pelvic organs and the key components of pelvic floor disorders.

Pregnancy and delivery are crucial contributors in the pathophysiology of incontinence and POP. The most common cause of AI in women is OASI which occurs in 1-11% of deliveries (Dudding et al. 2008; Laine et al. 2009). OASI increases the risk for AI two-fold compared to a non-complicated vaginal delivery at 18 years after delivery (Soerensen et al. 2013). UI affects 25-48% of women (Hannestad et al. 2000; Schreiber Pedersen et al. 2017) although higher prevalence rates have been reported as well (Hunskaar et al. 2003). Also the prevalence of UI increases with pregnancy and delivery (Peyrat et al. 2002). Aging is another acknowledged risk factor for pelvic organ disorders (Wu et al. 2017). The incidence of POP surgery ranges from 1.5 to 1.8 per 1,000 women years and peaks in women aged 60-69 (Barber and Maher 2013). Olsen et al. found in 1997 that almost 30% of the patients are re-operated for either POP or SUI. In Finland the lifetime risk for an operation for SUI is 6% and for POP 13% (Kurkijärvi et al. 2016; Kurkijarvi et al. 2017). Recurrent prolapse is usually corrected with artificial mesh material and significant controversy has arisen from the complications posed by the mesh surgery. Vaginal mesh exposure is one of the most common complications with a mean incidence of 10.3% (Abed et al. 2011). Most exposures are treated conservatively, but larger defects with infection, bleeding or other symptoms usually need surgical intervention and resection of the mesh.

2.2 Anal incontinence

2.2.1 Anal continence and the anal sphincter complex

Maintaining anal continence involves a complex mechanism of anorectal function, colonic transit, stool consistency and volume. The internal and external sphincters and the puborectalis muscle work together to form the anal sphincter mechanism. The physiology of continence and defecation requires coordination of the pelvic

floor, the anal sphincter muscles and intact somatic and autonomous nervous system (Guillaume et al. 2017).

It is estimated that 75-80% of resting anal pressure comes from the internal anal sphincter (IAS) (Frenckner and Euler 1975). In humans, the IAS is a 0.3-0.5 cm thick expansion of the circular smooth muscle layer of the rectum. The remaining anal resting pressure comes from the external anal sphincter (EAS) and anal cushions. The contraction activity is caused mainly by EAS, which is a 0.6-1.0 cm thick expansion of the levator ani muscles. It consists of skeletal muscle fibers and is both consciously and unconsciously contracted. (Rao 2004) The conscious contractions are controlled by the somatic nerve fibers of the pudendal nerve whereas the unconscious contractions might be due to the smooth muscle fibers in the EAS and be controlled directly by the fourth sacral plexus (Shafik et al. 2014). The puborectalis muscle is the third important muscular component. It creates a forward pull and reinforces the anorectal angle (Parks et al. 1966).

2.2.2 Anal incontinence

AI is defined as an involuntary loss of flatus, liquid or solid stool that is a social or hygienic problem (Haylen et al. 2010a; Sultan et al. 2017). FI is a complaint of involuntary loss of solid or liquid feces and double incontinence a complaint of both AI and UI. New definitions also include the term passive fecal leakage which is involuntary soiling of stool without sensation or difficulty wiping clean and the term overflow fecal incontinence as seepage of stool due to fecal impaction (Sultan et al. 2017).

AI is often underreported and patients may find it hard to discuss with healthcare professionals. In a Finnish study, Aitola et al. found that only 27.2% of subjects who in a postal inquiry reported FI at least twice a month had discussed the problem with their physician (Aitola et al. 2010). Seven years later 58% of the subjects were still incontinent and many patients did not receive any treatment, or the management of FI was not satisfactory (Lehto et al. 2013). In the study of

Boreham et al., 28.4% of women seeking gynecologic care for various reasons were affected by AI (Boreham et al. 2005).

The sphincter can be injured in three different ways during the delivery: through a direct trauma, via nerve distention that causes neurological injury or by a combination (Healy et al. 2008; Weledji 2017). Obstetric anal sphincter tears are usually called injury (OASI, OASIS) or rupture (OASR). Obstetric damages to the birth canal can be classified into four categories depending on the severity of extension. The first degree injury is to perineal skin only; the second to the perineum involving perineal muscles but not the anal sphincter; the third degree involving the anal sphincter complex and the fourth degree extending to the anal mucosa. The third degree is subdivided into 3a) <50% of the EAS torn, 3b) > 50% of the EAS torn and 3c) both EAS and IAS ruptured. (Villot et al. 2015; Thiagamoorthy et al. 2014)

Women who sustain a third and fourth degree anal sphincter tear are at increased risk of subsequent FI compared to women without an obstetric tear (Fitzpatrick and O'Herlihy 2005; Borello-France et al. 2006). AI is observed in 2-28% of parous women at 2-12 months after vaginal delivery (LaCross et al. 2015). Johannessen et al. evaluated the prevalence and predictors of AI in late pregnancy and one year after delivery, and found that 25% of primiparas experience AI already in late pregnancy. They also found that anal sphincter injury was a significant predictive factor for AI. Older age and operative vaginal delivery were associated with anal urgency (Johannessen et al. 2014). Nordenstam et al. found that at 10 years after the first delivery, 57% of women with and 28% of women without OASI suffered from AI symptoms (Nordenstam et al. 2009).

The risk factors for third and fourth degree injuries include nulliparity, large birthweight, duration of the second stage of labor, assisted vaginal delivery (vacuum or forceps extraction), shoulder dystocia and persistent occipitoposterior position of the fetus (de Leeuw et al. 2001; Burrell et al. 2015; Gauthaman et al. 2016). The role of episiotomy is contradictory, many studies have reported it to be protective (Christianson et al. 2003; Eskandar and Shet 2009; Samarasekera et al. 2009; de Leeuw et al. 2001). Verghese et al. found in their systematic review that an accurately performed mediolateral episiotomy is protective against OASI, especially in nulliparous women (Verghese et al. 2016). On the other hand, especially an episiotomy angled closer to midline has been found to be a risk factor for OASI

(Andrews et al. 2006b). The induction of labor, epidural analgesia and maternal age have also been controversial in different studies (Eskandar and Shet 2009; Bowling et al. 2009).

There are two alternative surgical methods for the primary repair of a ruptured anal sphincter: the end-to-end and the overlapping techniques. In the end-to-end technique the torn ends are approximated and sutured (Abbott et al. 2010). In the overlapping technique the torn ends of the EAS are brought together overlapping one end over the other (Sultan et al. 1999). The superiority of either technique has been discussed widely. The Cochrane database systematic review in 2013 concluded that short term (6 to 12 months) results might be favorable towards immediate primary overlapping technique in terms of the AI and fecal urgency. However, at 36 months follow up there appears to be no difference between these two techniques (Fernando et al. 2013). Prophylactic use of antibiotics and laxatives are recommended during the immediate recovery (Buppasiri et al. 2014; Mahony et al. 2004).

2.2.3 Diagnosis and evaluation of persistent AI

Follow up of OASI-patients is widely recommended. Especially patients with symptoms of impaired continence should be followed up in a specialized multidisciplinary perineal trauma clinic (Dudding et al. 2008). Different evaluation forms have been used in order to find out AI symptoms: the Wexner Incontinence Score (WIS) (Jorge and Wexner 1993), the Pescatori score (Pescatori et al. 1992), the Fecal Incontinence Severity Index or the Rockwood scale (Rockwood et al. 1999; Rockwood et al. 2000), the St Mark's scale (Vaizey et al. 1999) and the Fecal Incontinence Questionnaire (Reilly et al. 2000). Also, a simple visual analogue scale (VAS) score has been tested to evaluate the bother of AI (Devesa et al. 2013; Paka et al. 2016).

Clinical examination includes inspection of the anal area, perineal skin reflex and digital examination. The resting and squeezing pressures are assessed by digital

palpation of the sphincter. However, clinical assessment has poor sensitivity for detecting anal sphincter defects (Roos et al. 2012). Gynecologic examination and exclusion of pelvic organ prolapse and especially rectocele and rectal intussusception is very important in evaluating the main cause of AI and estimating the treatment options (Hayden and Weiss 2011).

Endoanal ultrasound (EAUS) is more sensitive than clinical examination in detecting sphincter defects (Sultan et al. 1993; Thakar and Sultan 2004; Stoker et al. 2002). Both EAUS and endoanal magnetic resonance imaging (MRI) are sensitive tools for preoperative assessment, and both techniques can be used to track down surgically repairable anterior EAS defects (Dobben et al. 2007). Defecography is a dynamic fluoroscopic examination that can be used to evaluate the posterior compartment and its disorders: rectocele, enterocele, rectal intussusseption, rectal prolapse and pelvic floor decent. However, anal sphincter defects are more accurately diagnosed by EAUS than conventional or MRI defecography (Vitton et al. 2011).

Anorectal manometry (ARM) is a functional test that assesses the tone and the function of the anal sphincter muscles. It is useful in evaluating AI and constipation (Van Koughnett and da Silva 2013). The ARM can provide useful information about the resting anal pressure and the squeezing ability of the anal sphincter (Chaliha et al. 2007).

The pudendal nerves innervate the external sphincter bilaterally. The pudendal nerve terminal motor latency test is also used for measuring the function of the anus and perineum. However, the pudendal nerve terminal motor latency is not helpful in understanding the AI of an individual patient (Hill et al. 2002; Cooper et al. 2016). Electromyography (EMG) can also be used to assess the contraction of the external anal sphincter. EMG measures depolarization strength (not latency time), and the activity of both the external anal sphincter and puborectalis muscle is captured. EMG can be analyzed by needle, surface or anal plug techniques and the electrostimulation can also be used as part of the biofeedback therapy (Boselli et al. 2010).

2.2.4 Treatment options for persistent AI

Rehabilitation of the pelvic floor after delivery, and especially after OASI, is widely recommended and augmented biofeedback physiotherapy is a gold standard treatment after OASI (Fitzpatrick and O'Herlihy 2005; Brincat et al. 2015).

For the patients with persistent AI symptoms or AI without a recognized predisposing trauma the first line treatment is conservative. It is important to acheive a balance between adequate fiber intake and stool consistency. Supplementation with dietary fiber containing psyllium or gum arabic was associated with a decrease in the percentage of incontinent stools and an improvement of stool consistency (Bliss et al. 2001). Medication such as loperamide, diphenoxylate and atropine are useful in influencing stool solidity. There is also evidence that loperamide might have positive impact on internal anal sphincter activity and resting pressure (Omar and Alexander 2013). A combination therapy with biofeedback and medical treatment has been effective for symptom relief. Symptom improvement was associated with improved fecal consistency, reduced urgency, and increased rectal sensory thresholds. (Sjodahl et al. 2015)

If the OASI primary repair and conservative methods fail, a secondary repair sphincterorraphy might be indicated for localized sphincter ruptures (Meurette et al. 2014). The overlapping technique for anal sphincter secondary repair involves identifying the fibrotic tissue and both ends of the external anal sphincter, mobilizing and suturing (Parks and McPartlin 1971). The role of secondary sphincteroplasty has been questioned because success rates are low in long term, especially for patients with pudendal neuropathy (Altomare et al. 2010).

Nowadays preferable methods for the treatment of AI include transcutaneous electrical nerve stimulation and sacral nerve stimulation (SNS), also known as sacral neuromodulation (SNM), which was first reported to treat FI by Matzel et al. (Matzel et al. 1995). Hull et al. found that 89% of patients maintained a significant improvement (≥50% less symptoms) and 36% had complete continence at five years after SNS in a selected patient group (Hull et al. 2013).

An artificial anal sphincter is a device with the intent of surgically augmenting the function of the anal sphincter complex. A systematic review found, however, that the need for surgical revision of this devive increases as continence decreases with time. Since the commercialization of SNS, artificial sphincter implantation has been limited (Hong et al. 2013). The magnetic anal sphincter is composed of a magnetic bead that creates a negative pressure around the anal canal. Wong et al. demonstrated that the magnetic sphincter is as effective as SNS in improving the symptoms and QoL in patients suffering from FI (Wong et al. 2012). Graciloplasty is a technique of muscle transposition aiming to replace anal sphincters, especially in cases where sphincter damage is too severe. Dynamic graciloplasty with a neurostimulation device significantly improves QoL and AI for some patients, but the five year results are modest and the complication rate high (Thornton et al. 2004). A colostomy increases QoL of incontinence patients and offers definitive treatment for individuals with severe FI when other treatment options have failed (Colquhoun et al. 2006).

Injectable bulking agents are materials designed to narrow the anal canal to improve anal continence. The studies conducted are mostly small, the effect on functional parameters such as anal contraction pressure is not clear and the application of the materials is limited by reabsorption, delayed foreign body reaction and possible migration. However, several bulking agents have been injected either into the submucosal or intersphincteric space. Most trials have reported a short term benefit regardless of the material used, including placebo saline injections (Maeda et al. 2013). The bulking agents used include collagen (Stojkovic et al. 2006; Maslekar et al. 2013), NASHA Dx (dextranomer in stabilized hyaluronic acid) (Graf et al. 2011), silicone and carbon-coated beads (Morris et al. 2013). Submucosal injections of polyacrylamide hydrogel Bulkamid® was recently used to treat AI and a modest although statistically significant overall improvement in AI symptom scores with corresponding improvements in QoL was observed (Altman et al. 2016).

2.3 Urinary incontinence

2.3.1 Urinary continence mechanism

Normal function of the urinary system is the result of balance between the urine secretion of the kidneys, the adequate storage system of the bladder and the voluntary control of emptying the bladder. Female urinary continence is maintained by the urethral sphincter and the vaginal support system. During bladder filling the urethra should provide a tight seal. The female urethra is a multilayered structure consisting of striated muscle, smooth muscle, connective tissue, submucosal vascular plexus and epithelium. Striated muscle has been shown to be responsible for one-third of the total urethral pressure, another third is exerted by the urethral vascular bed, and the remaining third is controlled by the smooth musculature and connective tissues (Delancey 2010; Rud et al. 1980).

2.3.2 Urinary incontinence

UI is defined as involuntary loss of urine. The most common type of UI is stress urinary incontinence (SUI) and it is defined as involuntary loss of urine upon the effort of physical exertion. Other types are urgency urinary incontinence (UUI) and mixed urinary incontinence (MUI), which is a combination of the two aforementioned types. UUI is incontinence associated with the feeling of urgency that is a complaint of a sudden, compelling desire to pass urine which is difficult to defer (Haylen et al. 2010a). SUI can be further categorized as urethral hypermobility, intrinsic sphincter deficiency (ISD), or both (McGuire et al. 1976). Risk factors for UI are obesity (Uustal Fornell et al. 2004; Danforth et al. 2006), advancing age (Zhu et al. 2009), parity (Rortveit et al. 2001), vaginal delivery (Altman et al. 2006) and diabetes (Danforth et al. 2009). The effect of hysterectomy is controversial (Gustafsson et al. 2006; Heliovaara-Peippo et al. 2010). Recently Kocaay et al. found hysterectomy for benign gynecological indications to have a negative impact on the pelvic floor function and to increase

the prevalence of pelvic floor disorders including UI, although the study did not have a control group and the effect of aging is likely to be a confounding factor (Kocaay et al. 2017).

2.3.3 Diagnosis and treatment options of SUI

Anamnesis is the essential part in distinguishing SUI, MUI and UUI. Disease-specific validated questionnaires are often useful. The Urinary Inventory Severity Score (UISS), the Incontinence Impact Questionnaire short form (IIQ-7) and the Urogenital Distress Inventory short form (UDI-6) assess the impact of UI on patients QoL (Uebersax et al. 1995; Stach-Lempinen et al. 2001). The Detrusor Instability Score (DIS) helps to differentiate between SUI and UUI (Klovning et al. 1996). Bladder diary is helpful in recording the times of micturition, the voided volumes, pad usage and fluid intake (Bright et al. 2014).

Gynecological examination with the ultrasonographic residual measurement and the cough test are the basic elements of differential diagnostics. The cough test is a provocative stress test that aims to raise the intra-abdominal pressure. The idea is to observe whether there is a leakage of urine during or after the coughing. The cough test is a reliable method for diagnosing SUI (Nager 2012). Recently Patnam et al. postulated that the cough test should be performed standing if the standard supine test is negative (Patnam et al. 2017).

The urethrocystometry (UCM) is a test that provides a subjective interpretation of an objective measurement of pressures of urethra (urethral profilometry, maximal urethral closure pressure), urinary bladder pressure during filling, pressure during exertion (urethral closure pressure) and voiding (pressure flow urodynamics). Urodynamics is often used for determining the type of incontinence (SUI from MUI), the pressure profile of the urethra (to distinguish the two types of SUI; urethral hypermobility and ISD), and to detect voiding dysfunction. It is also used to attempt to quantify the severity of incontinence (Patel and Chapple 2008a). However, it seems that the urodynamic evaluation does not result in better

outcomes in SUI surgery (Clement et al. 2013). Pad tests have been used for analyzing the severity of UI. However, there are no standardized protocols for pad tests and recently Krhut et al. showed that there is no linear correlation between the pad test and the severity of UI (Krhut et al. 2018).

Conservative treatment is the first line option for any type of UI. Drinking habits, dietary elements, bladder control and defecation habits, weight reduction and pelvic floor exercise are the main cornerstones in rehabilitating the pelvic floor function (Lukacz et al. 2017). Often conservative means fail to improve patient's QoL and further treatment options need to be taken into consideration.

A plethora of surgical techniques has been introduced to treat female SUI. One of the most widely used techniques has been the Burch colposuspension (Burch 1961). Midurethral slings have nowadays become primary surgical treatments for SUI because they are minimally invasive and have excellent short term and good long term success rates (Virkud 2011). The objective and subjective cure rates seem to be high (85-86% and 92-94% respectively) in both retropubic and transobturator tape operations five years after the procedure (Laurikainen et al. 2014). The cure rate seems to sustain during the long term follow up (Nilsson et al. 2008; Nilsson et al. 2013; Holdo et al. 2017; Heinonen et al. 2013).

Bulking agents are a treatment option especially for patients who are unresponsive to traditional treatments or who are not suitable for surgical treatment. Various substances, both non-degradable and biodegradable, have been used for injection material. However, injections with bulking agents have not been as effective as surgical methods, and the main problem has been the relatively poor sustainability of the bulking effect (Kirchin et al. 2012). The short term subjective cure rate with plain collagen (Contigen®) has been low in women with severe SUI (Groutz et al. 2000). Several investigations have produced variable results from 23% up to 83% cure rates at 1 and 2 years of follow up after collagen therapy (Corcos et al. 2005). Polyacrylamide hydrogel (Bulkamid®) is registered for transurethral injections for treatment of SUI. Subjective cure rate in a multicentre study with a two-year follow up was 64%; the objective cure rate was not measured (Toozs-Hobson et al. 2012). The report of 500 cases of bulking agent treatments (collagen, hyaluronic acid, ethylene vinyl alcohol, polyacrylamide hydrogel) showed improvement in subjective and objective outcomes in elderly patients and, in

contrast to earlier reports, side effects due to injections have been shown to be few and mild (Mohr et al. 2013).

2.4 Other gynecologic disorders requiring reconstruction

2.4.1 Pelvic organ prolapse and epithelial defects

Synthetic materials have mostly been used for recurrent POP to prevent further relapse. The most common and best documented material for transvaginal mesh surgery is non-degradable polypropylene mesh (Maher et al. 2013). However, the risk of vaginal exposure is up to 19% (Nieminen et al. 2010) and due to various complications the FDA has provided strict guidelines for mesh application and patient selection (www.fda.gov). The laparoscopic approach has become increasingly popular in POP surgery, but studies about the ideal method and route (vaginal vs. laparoscopic) are heterogeneous and so far no ideal method has been found (Maher et al. 2013). Therefore, there is a need for biodegradable engineered material that would help to prevent POP recurrence, to possibly remediate complications caused by synthetic mesh erosions and to create sustainable tissue for reconstruction. Large epithelial defects that require the use of biomaterial may also occur after extensive cancer operation, especially pelvic excenteration (Bacalbasa and Balescu 2015; Yin et al. 2013).

2.4.2 Congenital malformations

Patients with congenital malformations of the genitourinary tract and reproductive organs often need extensive surgical treatment. Cloacal and bladder extrophy are extreme examples of failed embryonal fusion in the midline which can cause various genitourinary malformations, e.g. uterine deformities (uterus unicornis, bicornis, didelphys) and absence of uterus and vagina (Morcel et al. 2007). The

most common cause of vaginal agenesis is the Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome, which is a multifactorial genetic syndrome with a normal 46XX-karyotype and normal female secondary sex characteristics. The incidence is estimated to be one in every 4000-5000 births (Aittomaki et al. 2001). Failed midline fusion during fetal life leads to the absence of vagina combined with uterine anomalies, and, in more than 1/3 of cases, renal and skeletal anomalies (Oppelt et al. 2006).Vaginal agenesis is mostly treated with conservative therapy using vaginal dilatation techniques, and anatomic and functional success rates of up to 90% have been achieved (Roberts et al. 2001). Surgery is considered when the noninvasive method fails or if the patient refuses to undergo dilatation. Numerous graft materials, including skin grafts, myocutaneous flaps, buccal mucosa grafts, bowel substitution, and peritoneum have been investigated (Callens et al. 2014). Additionally, techniques such as tissue expansion vaginoplasty and surgical modification of the dilation method (Vecchietti) have been used (Thomas and Brock 2007).

2.4.3 Transgender reconstruction

Gender dysphoria (transsexualism) is a condition in which the person´s gender identity – a sense of being a man or a woman - contradicts his or her bodily sex characteristics (Dhejne et al. 2011). Since the terminology and diagnostic criteria have changed over the years and cultural, social and religious factors might affect reliability of the methods, it is difficult to estimate the true prevalence of gender dysphoria. The incidence is also affected by legislative factors in different countries and during various periods of time. According to Arcelus' review, the overall prevalence of gender dysphoria is 4.6 per 100,000, varying from 0.40 per 100,000 (USA, approximation) to 23.60 per 100,000 (Singapore, sex reassignment surgery) (Arcelus et al. 2015).

Transgender patients undergo a series of multidisciplinary assessments for the appropriate diagnosis before hormonal treatment is started. Transgender reconstructive surgery is a fairly new and challenging field of surgery. For female to male transgender patients, the surgical treatment includes mastectomy, removal of

the uterus, ovaries and fallopian tubes and the vagina in order to be able to proceed to the phalloplasty, or metoidioplasty, which is an operative enlargement of the clitoris (Sutcliffe et al. 2009). External genital reconstruction requires advanced techniques and often the most difficult part is the reconstruction of the urethra.

2.5 Tissue engineering

Tissue engineering and regenerative medicine is a multidisciplinary field of science that has been developing rapidly during the last several decades along with the development of biotechnology. Advances in cell biology, molecular technology, bioengineering and material technology and even gene technology have helped to develop novel techniques and promising treatment methods for various conditions. The goal of tissue engineering is to repair or replace damaged tissues or organs by delivering cells, scaffolds, DNA or proteins at surgical procedures (Butler et al. 2000). Tissue engineering utilises cells, various kinds of biomaterial and growth factors to promote tissue healing and cell differentiation (Figure 2) (Toda et al. 2007).

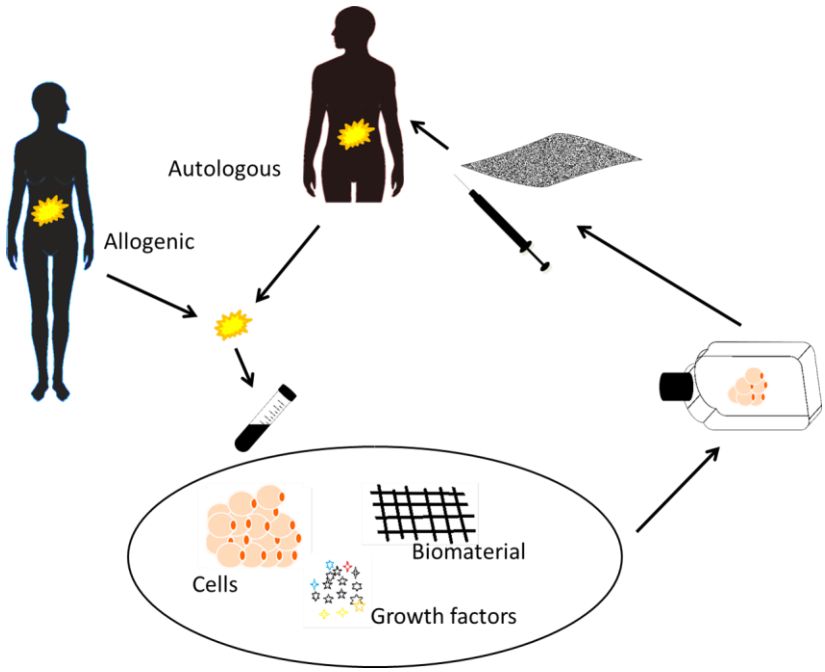

Figure 2. A schematic drawing of the three components of tissue engineering: cells, biomaterials and growth factors.

2.5.1 Cell sources for tissue engineering

Both stem cells and primary cells can be used for tissue engineering purposes. Stem cells are undifferentiated cells that are capable of proliferating indefinitely and able to differentiate into several pathways during the entire life of an organism. When the cells divide, the newly formed cells either remain a stem cell or differentiate for example into muscle, blood, epidermal or adipose cells (Lo Furno et al. 2016). Ideally, the cell source for tissue engineering purposes should fulfill the following criteria: availability in abundant quantities; possibility to be harvested from a suitable donor by a minimally-invasive procedure; controllable potential for differentiation along multiple cell lineage pathways; safe and efficacious transplantation and the potential to be manufactured in accordance with Good Manufacturing Practice (GMP) (Gimble et al. 2007).

Stem cells can be classified according to their differentiation potential (Keller 2005). Totipotent stem cells have the highest differentiation potential and they can be directed into any tissue type. Embryonal cells in zygote and morula stage are totipotent stem cells. Pluripotent stem cells can differentiate into cells from three different germ cell layers - endoderm, mesoderm and exoderm - but not into extra-embryonic tissue (Itskovitz-Eldor et al. 2000). Embryonic cells from the inner cell mass of the blastocyst and induced pluripotent stem cells are considered to be in this category. Multipotent stem cells are capable of self-renewal and have the potential to differentiate into organ-specific cell types, for example mesenchymal stem cells (adipose, umbilical, bone-marrow stem cells), hematopoietic stem cells or neural stem cells (Ullah et al. 2015; Prodinger et al. 2017). Unipotent stem cells can give rise only to one type of cells, e.g. epithelial cells (Blanpain and Fuchs 2009). Induced pluripotent stem cells are a fairly new entity of differentiated cell that are reprogrammed, i.e., manipulated in the laboratory to express genes normally present only in embryonal stem cells. Induced stem cells can therefore differentiate into cells of all organs or tissues (Alwaal et al. 2015; Takashima et al. 2017).

Another classification can also be used for stem cells: categorization into embryonic and adult, i.e., non-embryonic stem cells. Studies of adult stem cells began more than 50 years ago, when two populations of stem cells in the bone marrow were discovered (Friedenstein et al. 1968). Hematopoietic stem cells are

the origin of circulating blood cells (Uchida and Weissman 1992). Bone marrow stromal cells (BMSCs) are stem cells for non-hematopoietic tissue (Prockop 1997). BMSCs and adipose stem cells (ASCs) are mesenchymal stem cells (MSCs). They have the ability to differentiate, e.g. into adipocytes, osteoblasts and chondrocytes. MSCs are present in most adult tissues, e.g. adipose tissue, amniotic fluid, dental tissue, peripheral blood, endometrium and menstrual blood, and synovial fluid (Wagner et al. 2005; In 't Anker et al. 2003; Huang et al. 2009; Ab Kadir et al. 2012; Schuring et al. 2011; Morito et al. 2008). MSCs are also present in various fetal tissues during pregnancy including placenta and umbilical cord blood (Raynaud et al. 2012).

MSCs were first isolated from bone marrow (Friedenstein et al. 1968) and it was the main source of MSCs for many years. Bone marrow samples are collected from the iliac crest in an operation which is invasive and painful for the patient. Also, the aspiration often results in low yields of MSCs and the sample requires multiple passages to reach a suitable amount of cells (Morcos et al. 2015).

ASCs were isolated in 1976 from human adipose tissue, but they were properly characterized in 2001 (Zuk et al. 2001). The advantage of ASCs is that the tissue can be easily harvested and is readily available in large quantities. Subcutaneous fat is an accessible source of both ASCs and the more heterogenous stromal vascular fraction (SVF) (Gimble et al. 2011). White fat is an important energy reserve in the body and for obese people up to 50% of the weight is adipose tissue (Roche et al. 2010). Adipose tissue can be retrieved by either liposuction or subcutaneous adipose tissue fragments. ASCs can be easily expanded in vitro and have extensive self-renewal capacity (Zuk et al. 2002).

The International Society of Cellular Therapy has defined criteria for the MSCs. Besides being plastic adherent in standard culture conditions the cells must express certain surface molecules in immunostaining. Cluster of differentiation (CD) proteins are surface proteins that are important in cell adhesion, signaling and immunological response. To be characterized as being from mesenchymal origin the cells must express CD105, CD73 and CD90 and lack the expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR in flow cytometry. The MSCs must also be able to differentiate in vitro into adipocytes, osteoblasts and chondroblasts (Dominici et al. 2006).

The mechanism of action of stem cell therapy can be a direct integration and differentiation into other cell types (e.g. muscle cells) or a paracrine function with trophic effect or immunomodulation (Lane and Jacobs 2012; Callewaert et al. 2017). MSCs have shown to be multipotent with the ability to differentiate into different cell types based on environmental conditions (Dimarino et al. 2013). The MSCs also produce molecules that have an effect on inflammation, immune defense and angiogenesis (de Girolamo et al. 2013). Recently, it has been suggested that the effect of MSCs might be mediated through extracellular vesicles secreted by the MSCs (Nooshabadi et al. 2018). BMSCs have widely been tested in preclinical studies for the treatment of AI or UI (Lorenzi et al. 2008; Salcedo et al. 2013; Salcedo et al. 2014; Mazzanti et al. 2016; Du et al. 2013).

Primary cell cultures contain cells isolated directly from the tissues. In contrast to stem cells, primary cells are differentiated cells that can be cultured in laboratory in an appropriate media suitable for the cell type, but which have a restricted growth potential. Vaginal epithelial and stromal cells (fibroblasts) utilized in study IV are examples of primary cells. Nowadays, it is also possible to purchase commercial primary cell lines for research purposes.

2.5.2 Biomaterials for tissue engineering

A biomaterial can be described as a material designed to interact with the biological environment to replace, treat or augment host tissue (Williams 2009). When designing biomaterials for tissue engineering, one must thoughtfully consider the interplay between the targeted cells/tissues and the material. Important factors include cell–material interactions and mechanical properties of the microenvironment (Lutolf and Hubbell 2005). A biomaterial for pelvic floor disorders should also be processible, flexible, elastic and resilient. For implantation a scaffold material should also be suturable and easy to handle during surgery.

Scaffolds are three-dimensionally structured biomaterials or matrices that have been engineered for medical purposes. A scaffold material should promote the growth of the cells and serve as extracellular matrix (ECM) to attach the engineered tissue firmly to the surrounding tissue. A scaffold should allow cell attachment and migration, deliver biochemical factors and enable diffusion of cell nutrients. Biomaterials can also be loaded with bioactive factors, such as cytokines or growth factors to support the growth and differentiation of the cells (Atala 2009; Eberli et al. 2009).

Most biomaterials are based on natural polymers such as collagen or synthetic polymers such as poly (-ε)-esters (Nair and Laurencin 2007). Both biologically derived natural polymers and synthetic polymers degrade either enzymatically or by hydrolysis. The biocompability of the material is affected by the material chemistry, molecular weight, solubility, shape and structure, hydrophilicity/hydrophobicity, lubricity, surface energy, water absorption, degradation and erosion mechanism (Nair and Laurencin 2007; Atala 2011; Pariente et al. 2001). Natural biomaterials have bio-properties that mimic native tissue ECM. Some of the disadvantages include limited supply and poor mechanical features. They may also cause immune response. Synthetic polymer-based scaffolds may be manufactured on a large scale with suitable features of micro-nanostructure, strength and degradation process. Additionally, there are composite scaffolds, which consist of both an ECM component, such as collagen, and a synthetic polymer, or two different synthetic polymers (Alberti 2016).

Several injectable materials have been tested for cell preservation and transportation to biological tissue (Table 1). Hydrogels are a class of materials with numerous advantages to simultaneously encapsulate cells and biomolecules, and numerous gel systems allow one to intimately control the release characteristics through systematic changes in the gel's physical and chemical structure (Lin and Metters 2006). Among examples of hydrogels are collagen and polyacrylamide hydrogel.

Collagen is the main component of connective tissue. There have been attempts to improve the stiffness of collagen by cross-linking and mechanical compression. Collagen has also been combined with other ECM-proteins, e.g. elastin. Bovine collagen has been used for injection therapy for many clinical conditions, including SUI (Dmochowski and Appell 2000; Davis et al. 2013). The product of Contigen®

is a purified derivative of bovine dermal collagen that is cross-linked with glutaraldehyde solution and phosphate-buffered saline (PBS). It contains 95% of type I collagen and 5% of type II. The resulting gel is low-viscous and non-pyogenic, and after injection the gel becomes colonized by host-connective tissue cells. In animal studies, there was a rapid invasion of fibroblasts into the injected gel, formation of host type III, IV and V collagen and slight inflammation after rat subcutaneous injection of collagen. The implant remained in animals without visible modification during the 90-day experiment (Vialle-Presles et al. 1989).

Polyacrylamide hydrogel is a nondegradable gel with 2.5% dry matter and 97.5% water. It does not contain microparticles but instead is slowly integrated into host tissue by vessel ingrowth. Long term risk of fibrosis and migration is very low (Christensen et al. 2008). Bulkamid® is registered for use in SUI as a bulking agent (Lose et al. 2006; Toozs-Hobson et al. 2012). It has also been tested for anal incontinence treatment in women (Altman et al. 2016).

Numerous scaffold materials have been used for replacement of damaged or undeveloped organs (Table 1). The first application of free tissue graft was performed by Neuhof in 1917 when fascia was used to augment bladders in dogs (Neuhof 1917). After that, several natural scaffolds such as bladder acellular matrix, small intestine submucosa and naturally-derived ECM components such as collagen, elastin and hyaluronic acid have been tested (Alberti 2016).

Synthetic materials polylactide, polyclycolide and polycaprolactone are examples of biomaterials used for urothelial tissue engineering (Atala 2009; Wunsch et al. 2005). A highly elastic poly-L-lactide-co-ε-caprolactone (PLCL) membrane has been successfully developed for urothelial tissue engineering (Sartoneva et al. 2011). The advantage of synthetic polymer-based scaffolds is that they can be mass-produced, and in production the micronanostructure, strength and degradation process can be modulated (Alberti 2016).

Table 1. Natural and synthetic biomaterials and examples of their translational use.

		Feature, advantage, disadvantage	Examples of clinical application (references)
1.	**Injectable/liquid biomaterials**		
a.	**Natural**		
-	*Collagen*	Protein, main component of ECM, provides structural support in tissue. Risk of allergic reaction 4%	Contigen® in SUI, ung reconstruction combined with elastin (Groutz et al. 2000; Dunphy et al. 2014)
-	*Elastin*	Protein, provides elasticity in ECM	Combined with collagen, e.g.Matriderm® (Demircan et al. 2015)
-	*Albumin*	Protein, main function to maintain the oncotic pressure of blood	Coating for titanium implants (Hohn et al. 2017)
-	*Fibrin*	Protein, coagulation cascade	Wound glue (e.g. Tisseel®), preclinical trial for tendon repair w MSC, ongoing clinical trial for AI (Lee et al. 2017; Park et al. 2016)
-	*Hyaluronic acid*	Carbohydrate, found in ECM and in interstitial gel	Bulking for UI, murine model for wound healing (Alova et al. 2012; Murphy et al. 2017)
-	*Chitosan*	Polysaccharide, hemostatic agent, obesity treatment	Dental implant (Govindharajulu et al. 2017), Celox™-haemostat

b.	**Synthetic hydrogels**		
-	*Polyacrylamide hydrogel*	Synthetic polymer of acrylamide, either straight-chain or cross-linked	Bulkamid® for SUI, AI (Toozs-Hobson et al. 2012; Altman et al. 2016). Contact lenses, arthrosis injection, cosmetic surgery
2.	**Scaffolds**		
a.	**Natural**	Acellular tissue matrices	
-	*Collagen (ECM)*	Human, porcine, bovine collagen	Pelvicol®, Permacol™ in POP-surgery, breast reconstruction surgery AlloDerm®, urothelial tissue combined with PLGA (Hviid et al. 2010; Jones et al. 2017; Nakanishi et al. 2003)
-	*Bladder submucosal acellular matrix*	Human, porcine, intact or lyophilized	Preclinical studies on bladder tissue engineering (Bolland et al. 2007; Coutu et al. 2014)
-	*Small intestine submucosa*	Human, porcine, intact or lyophilized	Tendon repair, Durasis™ neurosurgery (Malcarney et al. 2005; Barber et al. 2006; Bejjani et al. 2007)
-	*Fibrin scaffold*	Protein, coagulant	Bone tissue engineering, Tachosil® haemostatic (Noori et al. 2017; Kim et al. 2015)
b.	**Synthetic**		
-	*Polyglycolic acid (PGA)*	Fiber forming ability, excellent degradability, good mechanical properties, cell viability	Suture material Dexon®
-	*Polylactic acid*	Slow degradation, good tensile strength, low extension, high modulus	Orthopedic devices, e.g. BioScrew®, BioAnchor®; POP-surgery (Mangera et al. 2013)

-	*Poly(lactide-co-glycolide)(PLGA)*	Good processability, controllable degradation rate, good suture ability, good cell adhesion	Suture material Vicryl®, Vicryl Rapid®; POP-mesh Vicryl Mesh®, Dermagraft®;In vitro study w vaginal fibroblasts, pig urothelial cells (Hung et al. 2010; Nakanishi et al. 2003)
-	*Polydioxanone*	Slow degradation, good handling properties	PDS® absorbable suture
-	*Polycaprolactone*	Highly processible, cheap, slow degradation	Monocryl® absorbable suture, targeted drug delivery
-	*Poly-l-lactide-ε-co-caprolactone (PLCL)*	Synthesized from the monomers L-lactide and ε-caprolactone, elastic, soluble	Urothelial application, aortic aneurysm treatment (Sartoneva et al. 2012; Burks et al. 2006)
-	*Poly(trimethylene carbonate)*	Excellent flexibility, poor mechanical strength, in vivo degradation higher than in vitro due to enzymatic degradation	Maxon® absorbable suture
-	*Polyurethanes*	Good biocompatibility, flexural endurance, high strength, high abrasion resistance and processing versatility	Cardiac pacemakers, vascular grafts, catheters. Myocardial tissue engineering (Chiono et al. 2014)
-	*Polyvinylalcohol*	Excellent film forming, emulsifying and adhesive properties	Alloxan in diabetic wound (Chouhan et al. 2018)
-	*Polyethylene*	Good mechanical strength and biocompability	Dacron® mesh for abdominal hernia, XanoMatrix for MSC culture (Bhardwaj and Webster 2016)

2.6 Studies on anal incontinence and tissue engineering

2.6.1 Preclinical studies

There are several preclinical studies on stem cells in treatment of anal sphincter defects (Table 2). Rat models are perhaps the most common study designs. Most of the studies have used either MSCs or myoblasts as the cell origin. Functional anal sphincter measurements have been done mostly *in vitro* models using anal sphincter muscle samples from euthanized animals to measure the contractility after electrical stimulation (Kang et al. 2008; Lorenzi et al. 2008; Pathi et al. 2012; White et al. 2010).

Table 2. Preclinical studies on AI and tissue engineering/stem cell therapy.

Ref.	Cell source	Study groups	n, species	Outcome measure	Results	Possible mechanism, authors conclusion
(Kang et al. 2008)	Autologous MDSC	normal vs. cryoinjured vs. cryo+MDSC	15 rats	In vitro muscle contractility+ histology	Contraction amplitude ↑, PKH26+	Myofiber formation, injection of SCs improve function
(Lorenzi et al. 2008)	BMSC	sham vs. SR+saline vs. SR+BMSC vs. SR+BMSC+CsA	24 rats	In vitro contractility, muscle area	Improved contractility, muscle area↑	Formation of new myotubes and myofibers
(Aghaee-Afshar et al. 2009)	rabbit BMSC +hUCM	hUCM vs. BMSC vs. saline vs. medium vs. no treatment	25 rabbits	In vivo EMG, histology	Contraction↑, BrdU+, muscle tissue↑ after BMSC	Significant improvement with BMSC, mild improvement and more fibrosis after hUCM. New muscular tissue
(Kajbafzadeh et al. 2010)	rabbit muscle progenitor cells	muscle progenitor cells vs. saline	21 rabbits	In vivo ARM, EMG, histology	Resting tone↑, electrical activity, PKH26+	Myofiber formation ↑, less fibrosis
(White et al. 2010)	rat myoblasts (H9c2)¤	repair vs. no repair, SC-injection vs. buffer	120 rats	In vitro contractile function, histology	EFSr↑ with repair+SC	Myotube formation, paracrine growth factors
(Pathi et al. 2012)	rat BMSC	sham vs. im-BMSC vs. iv-BMSC vs. saline	224 rats	In vitro contractile function, histology	Contractility↑, collagen↑, TGF-β1+ LOX↑	Paracrine action, no cells visible after 7 days. No effect with iv-administration → no homing?
(Lane et al. 2013)	rat myogenic SC	myogenic SC vs. saline	32 rats	In vivo EMG, ARM	Resting +contraction amplitude↑	Trophic factors, healing↑

(Salcedo et al. 2013)	rat BMSC	SP vs. PNC vs. sham SP vs. sham PNC	70 rats	In vivo ARM, EMG	Resting anal pressure↑ in SP+MSC iv/im, not in PNC-group	trauma needed for MSC effect
(Salcedo et al. 2014)	rat BMSC	im. vs. iv. vs. no injury	50 rats	In vivo ARM, histology	Anal pressure↑, fibrosis↓, collagen↑	Better healing? Homing of SCs
(Oh et al. 2015)	Autologous myoblasts	injury vs no injury	15 dogs	In vivo ARM, histology	Resting pressure↑ contractility↑, PKH26+	”Bioactive contractile bulking agent”
(Fitzwater et al. 2015)	Myogenic SC (H9c2)	SC vs. PBS	40 rats	Histology alone: striated muscle volume, inflammation	No alteration	Other cellular process?
(Bisson et al. 2015)	Rat myoblasts	Myoblast vs. PBS vs. opposite site injection	11 rats	In vivo ARM + ES	Anal pressure↑, no effect with PBS or opposite site injection	Acute trauma needed for the effect
(Mazzanti et al. 2016)	rat BMSC vs, minimally manipulated MNC	sham vs SR	32 rats	In vitro contractility	Sphincter mass↑, EFSr↑	No differentiation, paracrine effect? minimally manipulated MNCs as effective as BMSCs

ARM=anorectal manometry, BMSC=bone-marrow mesenchymal stem cells, BrdU+=positive for bromodeoxyuridine labeled cells, Cr=cryoinjury, CsA=cyclosporine A, EFSr=electrical field stimulation response, EMG=electromyography, ES=electrostimulation, GFP=green fluorescence protein, hUCM=human umbilical cord matrix, LOX= lysyl oxidase, MDSC=muscle-derived stem cells, MNC=mononuclear cells, PBS=phosphate-buffered saline, PKH26+=positive for fluorescent dye with labeled cells, PNC=pudendal nerve crush, S=sphincterotomy, SR=sphincterotomy and repair, TGFβ= transforming growth factor β, ¤ commercial cell line

2.6.2 Clinical studies

There are a few clinical trials that have tested stem cell therapy for treatment of AI (Table 3). Muscle-derived stem cell injections have been used in small pilot studies in women and the results are promising. Frudinger treated 10 women with severe AI. Each woman underwent an anal electrical stimulation program before the cell treatment. The injections were performed under general anesthesia. The injections were placed at the anal sphincter ends and the scar tissue under ultrasound guidance. After 12 months WIS had decreased and QoL improved. The anal pressure increased at one and six months but the effect disappeared at the 12 month examination. The procedure was well tolerated and no adverse events were observed. The possible effect of the anal electrical stimulation could not be evaluated due to a lack of a control group. (Frudinger et al. 2010) At five years after the injections there was maintained improvement in incontinence episodes, physiological measurements of anal function and QoL (Frudinger et al. 2015).

Romaniszyn et al. published the results of an experimental pilot study, where 10 patients with AI due to various origins received autologous myoblast implantation in the external anal sphincter. Subjective improvement was seen in six out of nine patients at 12-month follow-up and significant sphincter function recovery was seen in five patients. EMG examination showed increase in signal amplitude at 18 week examination in all of the treated patients, but at 12 months two patients experienced deterioration of continence. (Romaniszyn et al. 2015)

Autologous myoblasts were used in a trial of Boyer et al. Intrasphincteric myoblast-injections were compared with saline-albumin-injection for 24 patients. The outcome measure was the Cleveland Clinic Incontinence score. The injections were well tolerated, safe and had some clinical benefit at 12 months despite a transient placebo effect at six months. (Boyer et al. 2017)

Sarveazad et al. recently published a randomized double-blind clinical trial utilizing hASCs in PBS and compared the injections with plain PBS. WIS was ≥8 before the treatment. All patients had external anal sphincter defect verified by EAUS. A sphincteroplasty operation was performed together with injection

treatment. Interestingly, there was no statistical difference in post intervention WIS scores between the groups. With EAUS, detected muscle occupied area was higher in hASC-treated group compared to the controls. Anorectal manometry was not performed. (Sarveazad et al. 2017)

According to clinicaltrials.com there is an ongoing trial using allogenic ASCs mixed with fibrin glue compared with ASCs in saline. The primary end point is safety. Efficacy is measured by WIS, ARM and EAUS. (Park et al. 2016)

Table 3. Clinical studies on stem cell injection therapies for the treatment of AI.

Ref.	Cell source	n, *follow-up*	Injection method	Outcome measures	Results	Authors conclusion
(Frudinger et al. 2010)	autologous MDSCs	10 female AI-patients, age 25-66, WIS ≥9; *12 months*	US-guided injection	Diary, WIS, ARM, QoL	WIS↓ 15.3 → 1.6 (mean), ARM↑ QoL↑	No morphological changes in US, possible placebo or bulking effect? Safe and mini-invasive treatment
(Frudinger et al. 2015)	autologous MDSCs	see above; *5 years*	US-guided injection	Diary, WIS, ARM, QoL	WIS↓ 15.3→0.7(mean), ARM↑ QoL↑	Higher anal pressures than at 1 year. Sustained improvement of QoL. No adverse events
(Romaniszyn et al. 2015)	autologous MDSC	10 (9 female, 1 male), age 20-68, WIS≥10, failed biofeedback; *12 months*	US-guided injections	ARM, EMG, WIS	ARM↑, EMG↑, WIS↓ in 6/9 patients	Treatment failures had poor EMG response →proper innervation needed for stem cell effect
(Boyer et al. 2017)	autologous myoblast	24 female age 25-64, CCIS≥10; *12 months*	US-guided injection vs. placebo	CCIS, QoL at 6 months	CCIS↓, QoL↑	Safe, good tolerance, clinical benefit
(Sarveazad et al. 2017)	allogenous ADSC	18 (15 female, 3 men), age 25-78, WIS≥8; *2months*	SP+ADSC vs. SP+PBS	WIS, EAUS, EMG	WIS↓ in both groups, EAUS muscle↑ after ADSC treatment	Muscle formation, less fibrosis. Heterogenic patient groups

ADSC=adipose-derived stem cells, ARM=anorectal manometry, CCIS=Cleveland Clinic Incontinence Score, EMG=electromyography, MDSC=muscle-derived stem cells, EAUS=ultrasonography, QoL=Quality of Life, SP=sphincteroplasty, WIS=Wexner Incontinence Score

2.7 Studies on urinary incontinence and tissue engineering

2.7.1 Preclinical studies

There have been a few preclinical studies in different animal models to test stem cell treatment for urinary incontinence (Table 4). The researchers have mainly used ASCs, BMSCs or MDSCs of animal origin in their UI-models.

The first studies in UI translational therapeutic techniques were conducted by Jack et al., who tested processed lipoaspirate from female patients by injecting the fluorescent labeled lipoaspirate cells into rat bladders and urethras. The injected cells were viable and incorporated into the recipient smooth muscle. There was also *in vivo* expression of α-smooth muscle actin as a sign of smooth muscle differentiation (Jack et al. 2005). Also, Rodriquez et al. reported that adipose-derived cells have the potential to differentiate into functional smooth muscle cells. Therefore adipose tissue is considered to be a usable source of cells for treatment of injured tissues where smooth muscle plays an important role (Rodriguez et al. 2006). The *in vitro* culture milieu is essential for the differentiation. Various physical stimuli can also be used for differentiation (Morcos et al. 2015).

Lin et al. caused SUI for rats by postpartum dilatation of the vagina and simultaneous ovariectomy. The adipose-derived stem cells (ADSCs) were isolated from the rat periovarian fat and transferred either by transurethral or intravenous injections. Transplantation of ADSCs with both urethral and intravenous injections was effective in the treatment and prevention of SUI compared to the saline controls (Lin et al. 2010). Wu transplanted ADSCs to treat pudendal nerve damaged SUI-rats. Transplantation of ADSCs significantly strengthened local urethral muscle layers and significantly improved the morphology and function of the rat urethral sphincter (Wu et al. 2011a).

BMSCs have also been tested for SUI treatment. Kinebuchi et al. evaluated the functional and histological recovery of urethrolysis and cardiotoxin injection

injured rat urethral sphincters after periurethral injection of autologous BMSCs or cell-free medium. There was a tendency for better recovery in the BMSC treatment group, but no significant effect was detected (Kinebuchi et al. 2010).

Lee et al. studied allogenic muscle-derived stem cells in a denervated female rat model and found that the MDSC-injections improved the leak point pressure (LPP) at one and four weeks after injection compared to the saline injections alone (Lee et al. 2003). In the study of Chermansky et al., ISD was caused for Sprague-Dawley female rats by cauterizing tissues lateral to the mid urethra. Treatment with MDSCs increased the LPP without affecting the bladder function compared to the salt solution control group. The MDSCs had integrated within the striated muscle layer and the MDSC-injected urethra was contiguous and had more nerves than the controls (Chermansky et al. 2004). Kwon et al. compared MDSCs and fibroblasts after bilateral sciatic nerve transection and found that both MDSCs and fibroblasts increased the LPP in a rat model, but only MDSCs significantly improved muscle strip contractility. A high-dose fibroblast injection caused urinary retention (Kwon et al. 2006).

Lee et al. also tested MDSCs versus bovine collagen injections in a pudendal nerve denervated rat model and found the injection of MDSCs to increase the urethral LPP and closing pressure. Collagen injections seemed to have beneficial effect at 4 weeks but not at 12 weeks, in contrast to the MDSCs' effect (Lee et al. 2004). Xu et al. tested MDSCs combined with fibrin glue in treatment of rat SUI. They noticed that fibrin glue may potentially improve the action of transplanted MDSCs in restoring the histology and function of the urethral sphincter (Xu et al. 2010).

Table 4. Preclinical studies on urinary incontinence and tissue engineering.

Ref.	Cell source	n, species	Injury, treatment	Outcome measure	Results	Authors' conclusion / possible mechanism of action
(Lee et al. 2003)	Allogenic rat MDSC	34 rats *	SND, sham vs. saline vs. MDSC	LPP	LPP↑ in MDSC group	Allogenic MDSCs improve the function, did not trigger immune response
(Chermansky et al. 2004)	Allogenic rat muscle-derived cells	25 rats	C	LPP	LPP ↑	Bulking effect? Neo-nerve growth?
(Mitterberger et al. 2008a)	Autologous myoblasts, PKH26-label	10 pigs	VUR	Histology	Cell survival, myofibre differentiation, in large amount necrosis, PKH26+	Differentiation+ in small amount, can´t be used as bulking
(Lin et al. 2010)	Autologous ADSC, EdU/BrDU-label	22 rats	VD, local vs. iv. ADSC vs. saline	Cystometry, histology	Less abnormal voiding in ADSC-groups, elastin ↑	Higher elastin and muscle content in rats with normal voiding pattern. ADSCs effective in treating or preventing SUI?
(Kinebuchi et al. 2010)	Autologous rat BMSC, GFP-label	25 rats	U+CT, sham vs. CFM vs. BMSC	LPP	No difference in LPP, GFP+	"Trend towards better recovery", no significant effect
(Xu et al. 2010)	Rat MDSC, fibrin glue, GFP-label	75 rats	PN, sham vs. PN vs. FG vs. MDSC vs. FG+MDSC	LPP, histology, IHC	LPP ↑ in FG+MDSC-group	Fibrin glue may potentially improve the action of transplanted MDSCs to restore the histology and function
(Wu et al. 2011a)	Rat ADSC	33 rats	PN, ADSC vs. controls	UCM, histology	BC ↑, LPP ↑, MUCP ↑, FUL↑	Improved function of sphincter, muscular structure

(Kim et al. 2011)	Allogenic MSCs	30 rats	PN, MSC vs. saline	in vivo LPP, CP	LPP ↑, muscle-spesific markers+	Improved function, MSCs differentiate into muscle cells
(Eberli et al. 2012)	Autologous muscle precursor cells,PKH26+	27 dogs	mS, muscle precursor cells vs. controls	Sphincter pressure, cystourethrogram, histology	LPP ↑, recovery of sphincter, myofibers, PKH26+	New muscle formation, nerve fibers
(Du et al. 2013)	BMSC, muscle-like cells	72 rats	PN, BMSC vs. muscle-like cells vs. calcium alginate gel vs. control	Myoblast formation, urethral resistance, LPP	LPP ↑, desmin, SMA ↑	Growth of blood vessels, formation of muscle?
(Silwal Gautam et al. 2014)	Autologous ADSC, PKH26	9 rabbits	C, ADSC vs. controls	LPP, muscle area	LPP ↑, Myoglobin, SMA	Bulking? Muscle area, growth factors
(Li et al. 2016)	ADSC (+3D microtissue)	48 rats	VD, ADSC vs. microtissue vs. controls	LPP, BC, histology, IF, IHC	LPP↑, BC↑	Biological factors

ADSC=adipose-derived stem cells, BC=bladder capacity, C=cauterization, CFM=cell free medium, CP=closing pressure, FUL=functional urethral length, IF= immunofluorescence, IHC=immunohistochemistry, mS=microsurgical sphincter removal, MSC=mesenchymal stem cell, PN=pudendal nerve cutting/transection, SMA=smooth muscle actin, SND=sciatic nerve denervation, U+CT=urethrolysis+cardiotoxin, VD=vaginal dilatation, VUR=vesico-ureteral reflux, *+ 4 additional rats for immunohistochemistry

2.7.2 Clinical studies

The clinical trials utilizing stem cells in treatment of SUI in women are summarized in Table 5. Most of the studies have used muscle-derived or myogenic stem cells. Collagen gel (Contigen®) was used in the studies of Mitterberger et al. as a cell carrier, saline in the study of Lee who used allogenic human umbilical cord blood SCs. Three studies did not report using any specific carrier with the cells (Carr et al. 2008; Sebe et al. 2011; Stangel-Wojcikiewicz et al. 2014). The studies are somewhat heterogenous, and different outcome measures were used. None of the studies reported using cough test as the primary outcome measure. QoL was measured using different evaluating methods. All of the studies have reported some beneficial effects of the injection treatment although comparisons between the studies or to controls cannot be made.

Table 5. Clinical studies in urinary incontinence and tissue engineering.

Ref.	Cell source, carrier	n, *follow-up*	Outcome measures	Results	Possible mechanism stated by authors
(Mitterberger et al. 2007)	Autologous myo- and fibroblasts*;Collagen (Contigen®)	123 female, *12 months*	IS, QoL¤ UCM, EMG	Median IS↓6 (5-6)→0 (0-4), contractility↑, thickness ↑	Regeneration of rhabdosphincter and submucosa
(Mitterberger et al. 2008b)	Autologous myo- and fibroblasts, Collagen (Contigen®)	20 female, *24 months*	IS, QoL¤, UCM, morphology +function of urethra	16/18 cured, IS ↓ 6→0, contractility↑, thickness↑, MUCP↑	Cells integrate and form myoblasts, stimulate the regenerative process
(Carr et al. 2008)	Autologous MDSC	8 female, *12 months***	Pad test, QoL¤¤	1 patient continent, 4 improved	Restoring muscle function
(Lee et al. 2010)	Allogenic hCBSC, saline	39 female, *12 months*	PST, UCM	"Cure 36%, improved 36%, failure 26%", MUCP ↑	Not discussed
(Sebe et al. 2011)	Autologous muscular progenitor cells	12 female, *12 months*	Flow, PVR, Diary, 1h pad, QoL¤¤¤	No change in Qmax, No retention; 3 patients improved, QoL ↑	No complications=safe. Efficacy? Suitable for recurrent UI
(Stangel-Wojcikiewicz et al. 2014)	Autologous MDSC	16 female, *24 months*	UCM, QoL¤¤¤¤	50% continent, 25% improved MUCP ↑	No complications=safe Effect of estrogen? (cured patients had longer time of exposure to estrogen)

Ct=cough test, EMG=electromyography, hCBSC=human cord blood stem cells, IS=incontinence score, MDSC=muscle-derived stem cells, MUCP=maximal urethral closure pressure, PST=patient satisfaction test, pt=patients, PVR=postvoidal residual, UCM=urethrocystometry.

*myoblasts were injected into rhabdosphincter and fibroblasts into urethral submucosa, **reinjections for 3 patients at 3 months,

¤ (Patrick et al. 1999), ¤¤ not specified, ¤¤¤ (Amarenco et al. 2003), ¤¤¤¤ (Gaudenz 1979)

2.8 Studies on other gynecologic disorders and tissue engineering

Tissue engineering techniques have been used to create replacements for congenital or acquired malformations of the urogenital tract. One of the first reports was from Atala, who reported having performed a full cystoplasty for seven patients with end-stage bladder disease. In their study urothelial and muscle cells were collected and cultured and a biodegradable bladder-shaped collagen scaffold was used to create a functional neobladder (Atala et al. 2006). Urethral replacement with cells acquired by a bladder biopsy seeded on a PGA-scaffold was studied in five boys having congenital urethral defects. Urethral biopsies showed a normal architecture of urethral mucosa at three months after transplantation. Also, the urethral function seemed normal according to cystourethroscopy and radiographic voiding cystourethrogram. (Raya-Rivera et al. 2011)

A total neovagina was developed for a rabbit model using small fragments of autologous vaginal tissue planted on PGA/PLGA-scaffolds. The tissue was well vascularized and the scaffold was completely degraded after 3 months (De Filippo et al. 2008). A bioengineered vaginal replacement was also created by the same group in an animal model using rabbit vaginal epithelial and smooth muscle biopsies and a PGA-scaffold (Dorin et al. 2011). The cell-seeded scaffolds were then implanted subcutaneously into immunocompromised athymic mice. After 1, 4 and 6 weeks the implants demonstrated the presence of vaginal epithelial and smooth muscle cells and complete layers resembling normal vaginal tissue. The group of Atala has also conducted a clinical study with four young (age 13-18 years) MRKH-patients with vaginal agenesis. Autologous vulvar tissue was cultured, expanded and seeded on PGA-scaffold (Raya-Rivera et al. 2014). Orabi et al. have created neovaginal tissue from vaginal biopsies without the use of exogenous material and grown the tissue successfully in nude female mice (Orabi et al. 2017).

PLGA has been tested for growing human vaginal fibroblasts in order to develop material for reconstruction of pelvic organ prolapse (Hung et al. 2010). In

this study, human vaginal fibroblasts were characterized with regard to their collagen content and proliferation potential, the fibroblasts were seeded on PLGA-scaffold. Histological features were assessed after planting the scaffolds subcutaneously in experimental, nude mice. The study revealed that 12 weeks after subcutaneous transplantation, neo-fascia was formed with complete resolution of the mesh. The newly formed fascia was either multi-layered or well-organized lamellar structure. The controls with collagen gel-coated PLGA did not form neo-tissue. Nakanishi et al. seeded porcine urothelial and smooth muscle cells on PLGA mesh collagen sponge hybrid scaffold and found that urothelial cells proliferated and formed layers. On the other hand smooth muscle cells failed to proliferate in PLGA collagen sponge but were successfully seeded in PLGA collagen gel. (Nakanishi et al. 2003)

Methoxypolyethylene glycol polylactic-co-glycolic acid, seeded with autologous muscle fiber fragments has recently been studied by Jangö et al. They found that biodegradable PLGA is suitable for fascia remodeling in a rat abdominal wall and that muscle fiber fragments might add some value in the mechanical properties. (Jango et al. 2017)

PLCL has not, to our knowledge, been previously studied for vaginal tissue engineering applications.

3 AIMS OF THE STUDY

In this thesis we wanted to evaluate the need for alternative treatments, develop novel treatment methods and utilize modern laboratory technologies in the treatment of pelvic floor disorders. The specific aims for different studies are elaborated below:

I. To assess the prevalence of AI symptoms six months after OASI primary repair, to identify risk factors for persistent AI symptoms, to evaluate the clinical usefulness of a simple three-choice assessment of AI symptoms and to evaluate the need for novel treatment methods (e.g. tissue engineering) for AI in these patients.

II. To test the efficacy of hASC injection therapy for treatment of AI and to evaluate a synthetic polyacrylamide hydrogel (Bulkamid®) as a carrier for hASCs, utilizing a rat model.

III. To assess transurethral injections of autologous ASCs combined with collagen (Contigen®) in treatment of female SUI to assess the safety and effectiveness of the method.

IV. To create a new combination of supercritical carbon dioxide foamed biodegradable PLCL-scaffold with epithelial and stromal cells isolated from vaginal tissue samples and to assess the suitability of this combined biomaterial for treatment of vaginal agenesis or other epithelial deficiencies *in vitro*.

4 MATERIALS AND METHODS

4.1 Subjects and study designs

4.1.1 Patients (I, III)

Study I was a retrospective study of an obstetric cohort of 325 patients with 3rd or 4th degree OASI who were operated on at a tertiary care teaching hospital in 2007-2013 (Figure 3). All patients were operated on within 24 hours after the sphincter injury. The patients were sent a questionnaire at six months about symptoms of AI. The subjective outcome was measured by WIS and an empirically developed three-choice assessment. The rationale of the three-choice assessment was to ask the patient if she 1) had no symptoms of AI; 2) had symptoms but did not wish for further examinations or treatment; or 3) had severe symptoms of AI necessitating further treatment. In order to examine variables associated with persistent AI, two groups were formed based on the three-choice assessment: those who reported no AI symptoms at 6 months after OASI (n=198) and those who had any minor or severe symptoms (n=112). The groups were compared regarding pre-labor and intrapartum associating factors (Table 6).

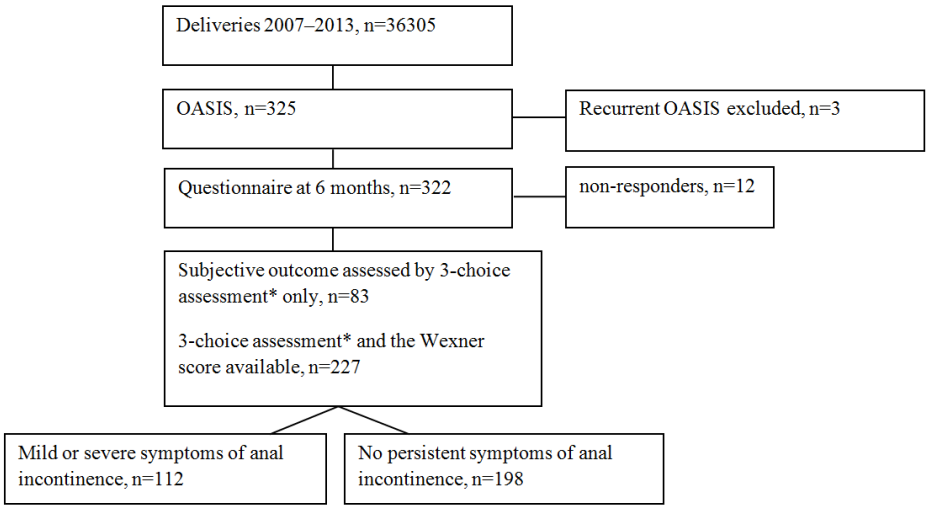

Figure 3. The patient flow chart of the Study I. * 3-choice assessment=no/mild/severe symptoms of AI. Copyright © Springer. Kuismanen et al. Outcomes of primary anal sphincter repair after obstetric injury and evaluation of a novel three-choice symptom assessment. Tech Coloproct. 2018 Mar;22:209-214. Reprinted with permission.

Study III was a prospective clinical pilot study. Patients were recruited from the Tampere University Hospital outpatient clinic. Five women with SUI or MUI who primarily did not want the mid-urethral tape operation were treated with transurethral injections of autologous ASCs and collagen to treat SUI. The isolation and expansion of ASC was done in a validated cleanroom (BioMediTech, University of Tampere) following European GMP quality system guidelines. The effect of the ASC-injections were evaluated by objective (cough and pad test, urodynamic measurements) tests and subjective validated questionnaires: Visual Analog Scale (VAS), Urinary Inventory Stress Test (UISS), Detrusor Instability Score (DIS), Incontinence Impact Questionnaire-short form (IIQ-7) and Urogenital Distress Inventory –short form (UDI-6). The women were followed up for one year (Table 6).

4.1.2 Animals (II)

Study II was a preclinical study utilizing an animal model for AI (Figure 4). Sixty female virgin Sprague-Dawley rats (age 12-14 weeks, Janvier laboratories) were anesthetized and the anal sphincter function was assessed by ARM before iatrogenic anal sphincter injury. The anal sphincter was cut to mimic grade 4 anal sphincter injury and sutured before the injection therapy. An optimal carrier material for stem cells was investigated by injecting human ASCs combined with either 0.9% NaCl (saline) or polyacrylamide hydrogel (Bulkamid®) or saline/hydrogel alone as controls after anal sphincter repair. The study was conducted at the premises of the University of Tampere under the approval of the Regional State Administrative Agency. The 3R principle of animal studies (replacement, reduction and refinement, see Chapter 6.6) was followed at the study design. The anorectal manometry, μCT, immunohistochemistry and histology of the sphincter area were detected to examine the effect of the stem cell injections. The groups were compared according to functional measurements, μCT and histological parameters (Table 6).

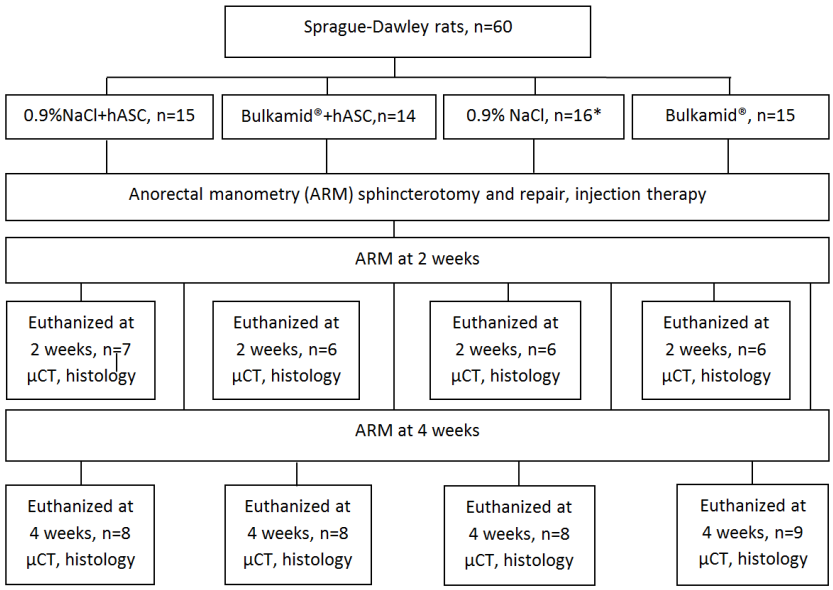

Figure 4. The flow chart of the Study II protocol. * 2 rats died presumably due to anesthesia complication. NaCl=sodium chloride; hASC=human adipose stem cells; ARM=anorectal manometry, µCT=micro-computed tomography. Copyright © Wiley Periodicals. Kuismanen et al. Functional outcome of human adipose stem cell injections in rat anal sphincter acute injury model. Stem Cells Transl. Med. 2018 Jan;7:295-304. Reprinted with permission.

4.1.3 Donors (II, IV)

In Study II, the hASCs were retrieved from abdominal subcutis of a female donor (age 67 years) in an elective plastic surgery operation at Tampere University Hospital (Tampere, Finland) with the patient's written consent. In Study IV vaginal epithelial and stromal cells were isolated from three transgender patients in their elective vaginectomy (removal of the vaginal epithelium) operation under informed consent, and expanded in cell culture. Isolated and expanded cells were

characterized using flow cytometry. Cells were combined with a novel porous scaffold material and cultured further up to 2 weeks. After this, cell-biomaterial combinations were analyzed (Table 6).

Table 6. Overview of the design of Studies I, II, III and IV.

	Study I	Study II	Study III	Study IV
Patients (n)	Women 6 months after OASIS repair (322)		SUI-patients (5)	
Animals (n)	-	Sprague-Dawley rats (60)	-	-
Cells	-	hASCs	Autologous hASCs	Vaginal epithelial cells + stromal cells
Biomaterial	-	Polyacrylamide hydrogel	Collagen	Foamed PLCL-scaffold
Examination	EAUS, ARM *	ARM	Gyn examination, Ct, UCM	-
Questionnaires	WIS, Three-choice assessment of AI symptoms	-	DIS, IIQ-7, UDI-6, UISS, VAS	-
Aquiring cells	-	From a human donor, elective plastic surgery	Subcutaneous fat, under local anaesthesia	From human donors (3), elective vaginectomy
Cell laboratory assays	-	L/D, FACS	L/D, FACS	L/D, FACS, PCR
Histology and immunohisto/cytochemistry	-	HE, Perls Prussian blue, Picosirius red, SMA, CD68,	α-SMA, SM22-α, MHCII,	Pancytoceratin, phalloidin
Imaging	-	µCT	Transvaginal US	µCT, SEM
Follow up	6 months*	2 and 4 weeks	1, 3, 12 months	7 and 14 days
Primary outcome measure	AI symptoms at 6 months	ARM: resting and contraction pressure	Cough test	Cell attachment, viability and phenotype
Secondary outcome measures	Risk factors for AI, correlation of WIS and three-choice assessment, mode of delivery in subsequent pregnancy	Continuity of muscle, visibility of PMP-50 labeled cells, inflammation	Subjective outcome, UCM	Tensile properties of scaffold

* Only patients with severe symptoms (n=27) were examined within 7-14 months.

CD68=Cluster of differentiation antibody used for rat macrophage detection, Ct=Cough test, DIS= Detrusor Instability Score, FACS=Fluoresence-activated cell sorter, HE=Hematoxylin & Eosin, IIQ-7= Incontinence Impact Questionnaire-short form, L/D=Live/dead analysis, MHCII=Myosin heavy chain II, PCR=Polymerous chain reaction, PLCL=Poly-l-lactide-ε-co-caprolactone, PMP-50=Magnetizable nanoparticles used for cell detection, SEM=Scanning electromictoscopy, SM22-α=Smooth muscle 22α, SMA=Smooth muscle actin, UCM=Urethrocystometry, UDI-6= Urogenital Distress Inventory-short form, UISS= Urinary Incontinence Severity Score, US=ultrasonography, VAS=Visual analogue scale, WIS=Wexner Incontinence Score, µCT=Micro-computed tomography.

4.2 Biomaterials (II, III, IV)

In Study II, hASCs were combined with either 0.9% NaCl or a synthetic polyacrylamide hydrogel Bulkamid®. Bulkamid® is a nondegradable viscoelastic water-based polymer and it is mainly used for injection therapy of female urinary incontinence (Christensen et al. 2008; Pai and Al-Singary 2015). The suitable amount of hASCs in combination with Bulkamid® and the injection technique was determined in a pilot study and the cell amount of 5 x10^5 hASCs was chosen for the anal sphincter injections.

Contigen®, a cross-linked glutaraldehyde collagen, is a bovine collagen product designed for UI injection treatment. It contains at least 95% type I collagen and 1% to 5% of type III collagen. Contigen® has widely been used for the treatment of SUI (Dmochowski and Appell 2000).

PLCL-scaffolds were manufactured at Tampere University of Technology, Faculty of Biomedical Sciences and Engineering. The material was produced by first melt-extruding PLCL polymer granules (polymer of 70/30 poly-l-lactide-co-ε-caprolactone) into rod shape with a customized twin-screw extruder. The rods were cut into smaller pieces, inserted into a mould and then foamed with supercritical CO2-foaming process (Barry et al. 2006). The foamed blocks were cut into the final disc shape by custom made blades with a diameter of 5mm and height of 2-2,5mm (Figure 5). The scaffolds were ethanol washed, vacuum dried and packed. The sterility of the scaffolds was achieved by gamma irradiation (minimum dose 25 kGy). The tensile tests were conducted to measure the elastic modulus in dry and wet samples (n=6).

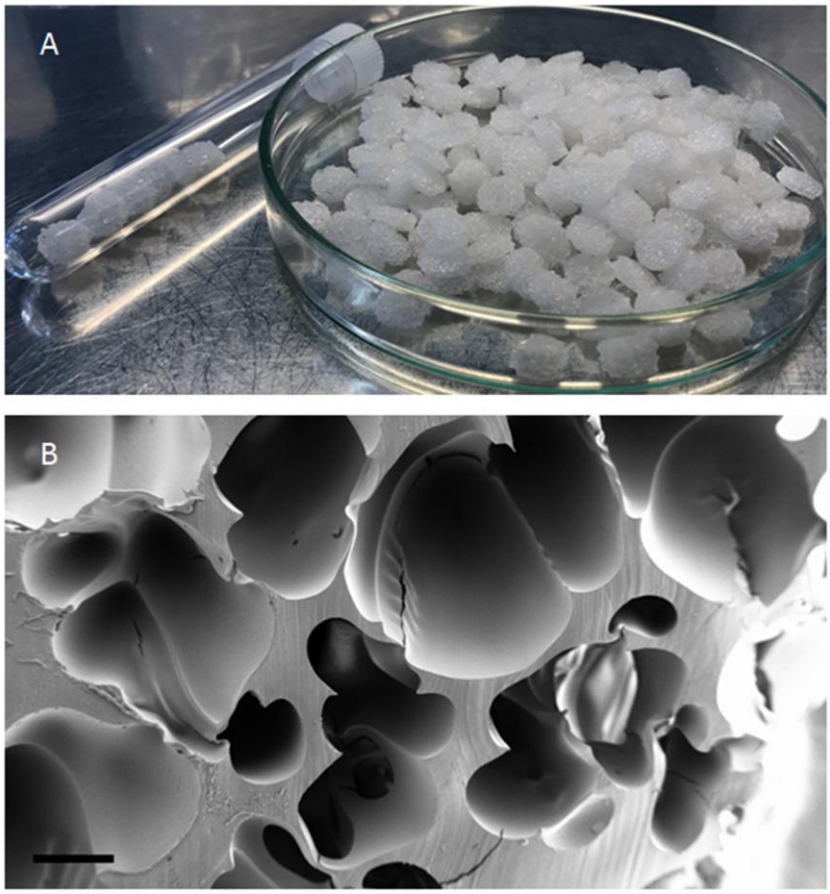

Figure 5. The scaffold material from study IV. A. Pieces of a supercritical carbon dioxide foamed PLCL-scaffold, diameter 5mm. B. SEM-figure of the same scaffold material. Scale bar 200µm.

4.3 Laboratory methods

4.3.1 Cell isolation and culture (II, III, IV)

In our studies the isolation and expansion of ASCs and vaginal epithelial and stromal cells were done at BioMediTech, University of Tampere. The cell isolation, expansion, karyotyping, sterility, endotoxin and mycoplasma testing are described separately in original publications. Briefly, the acquired adipose tissue (studies II, III) was minced and digested with collagenase. After centrifuging and lysing the red blood cells, the pellet was suspended in the basal medium. Then the isolated cells were expanded for 3-4 weeks. When nearly confluent (90%), the cells were mechanically detached using cell scraper and passaged. For the injection therapies in Study III, passages 3 to 4 were used, which were the lowest possible passages to get an adequate amount of cells. For the preclinical study (Study II), passages from 4 to 7 were used. For the vaginal epithelium (Study IV), the tissue was cut into pieces, digested and separated by centrifuging. The epithelial and stromal cells were cultured separately and passaged into cell lines (passage 2-3).

4.3.2 Cell viability and proliferation (II, III, IV)

Fluorescence staining was used to distinguish the living cells from the dead cells in a cell culture. Briefly, the cells were stained with Calcein AM/Ethidium homodimer -1-reagent and incubated. Live cells were distinguished by Calcein AM dye retained within live cells and they produce an intense uniform green fluorescence. The Ethidium-homodimer-1, on the other hand, enters cells with damaged membranes producing a bright red fluorescence in dead cells. A fluorescence microscope was used to image the viable and the dead cells. The material without cells (Study II Bulkamid®, Study III Contigen®, Study IV scPLCL scaffold) was used to exclude false positive staining by material. The DNA quantification was used to determine the density of cells in a culture. CyQuant®

Cell Proliferation Assay was used to monitor the adherence of cells to surfaces. The method uses green fluorescent dye which exhibits strong fluorescence enhancement when bound to cellular nucleic acids.

4.3.3 Cell characterization (II, III, IV)

In our studies, the ASCs were sorted using a fluorescence-activated cell sorter (FACS: FACSAria Fusion Cell Sorter, BD Biosciences). When the origin of the cells is determined, the viable cells should express certain molecules and lack the others. The minimal criteria for mesenchymal stem cell phenotype are the presence of CD105, CD73 and CD90 ($\geq$ 95%) and negative staining of CD45, CD34, CD14, CD11b, CD79α and CD19 ($\leq$ 2%) (Dominici et al. 2006). For the vaginal epithelial and stromal cells, no previous publications of the cell marker profile were available.

4.3.4 The biomechanical testing of the scaffold (IV)

The structure of the scPLCL scaffold was analyzed by μCT imaging. The porosity and pore size (Zeiss Xradia MicroXCT-400, XMReconstructor software and Avizo Software) were calculated. The elastic modulus was determined to assess the mechanical properties of the scaffold.

4.3.5 Imaging techniques (II, III, IV)

4.3.5.1 Histology (II) and immunohistochemistry (II, III, IV)

Histology was used as a method in Study II for analyzing the samples of the rat anal sphincter. The method is described in detail in the original publication. Briefly, after the fixation, the H&E-stained samples were assessed according to histologic parameters. Tissue reaction, inflammation and integration of Bulkamid® into the tissue were assessed (Nolte et al. 2016). Different staining methods and immunohistochemistry (Picosirius red, Perls Prussian blue, anti-human Vimentin, STEM21, smooth muscle actin and CD68) were used to detect and differentiate collagen, iron particles, human cells and rat macrophages. In Study III, the myogenic differentiation was assessed by immunostaining using smooth muscle protein α, α smooth muscle actin and myosin heavy chain II. In study IV, the expression of cytokeratin and organization of actin filaments were assessed in epithelial and stromal cell co-cultures at 7 and 14 day time points.

4.3.5.2 Micro-computed tomography (II, IV)

The rationale for μCT is to provide high-quality images of whole organs instead of sections or slices as in histology. It is also possible to perform structural analysis and volume calculations of the imaged tissues or samples of material. In our studies, μCT was used for visualization of the treated area of rat anal sphincter in Study II and to analyze the morphology of the porous scPLCL-scaffold in Study IV. In Study II, to enhance the contrast between soft tissues the samples were put through a staining regimen. Briefly, the paraformaldehyde solution used for fixation was rinsed with rising concentrations of ethanol and the samples were stained with iodine. After imaging 3D-images were reconstructed for further analysis. In Study IV the scPLCL-scaffolds were imaged to calculate the porosity and the pore size of the scaffold.

4.3.5.3 Scanning electron microscopy (IV)

In Study IV, the scPLCL-scaffolds seeded with epithelial and stromal cells were dried and gold sputtered. The SEM imaging was conducted at Tampere Technical University, with the scanning electron microscope Zeiss ULTRAplus.

4.4 The subjective cure assessments

4.4.1 WIS and the three-choice assessment (I)

In Tampere University Hospital over the past 10 years a three-choice assessment, together with the widely used WIS, has been used to detect patients who need and want further treatment six months after primary repair of OASI. WIS is a detailed questionnaire containing five questions about AI (Table 7).

Table 7. The Wexner Incontinence Score (modified from Jorge, Wexner 1993).

Type of incontinence	Frequency of the incontinence symptom				
	Never	Rarely	Sometimes	Usually	Always
Solid feces	0	1	2	3	4
Liquid feces	0	1	2	3	4
Gas	0	1	2	3	4
The use of pads	0	1	2	3	4
Lifestyle alteration	0	1	2	3	4

Rarely=less than once a month; sometimes=less than once a week; usually=less than once a day but more than once a week; always=more than once a day

The three-choice assessment inquired if the patient 1) had no AI symptoms; 2) had AI symptoms but did not want an appointment with a colorectal surgeon for the time being or 3) had AI symptoms that were disturbing necessitating further treatment.

4.4.2 UI QoL-questionnaires (III)

The subjective cure rate was assessed by validated questionnaires evaluating the QoL affected by the UI symptoms. UISS, IIQ-7 and UDI-6 are questionnaires directed especially to UI symptoms (Corcos et al. 2002; Uebersax et al. 1995). DIS evaluates the amount of urge symptoms (Kauppila et al. 1982). VAS, which originally was a validated tool for assessing pain, satisfaction and QoL and which is nowadays also used in urogynecologic research, was used to simply evaluate the patients' experience about the overall efficacy of the treatment (Lukacz et al. 2004).

4.5 Anorectal manometry (II)

In our animal study, ARM was performed using a Polygraf ID manometry system with triple lumen catheters (Polygram NET, Medronic, computer unit Windows XP). The measurements were conducted preoperatively and at 2 and 4 weeks after the operation. The duration of the ARM procedure was approximately 30 minutes, during which resting anal sphincter pressure and peak pressure during spontaneous contraction was measured 8-10 times. Mean values of these measurements were used for further analysis.

4.6 Cough test and urethrocystometry (III)

The cough stress test was performed for the SUI patients before the treatment and at 1, 3, 6 and 12 months control. The test was performed in lithotomy position with a "half-full" bladder of approximately 200-300ml of urine. The cough test was regarded as positive when urine was leaking after a few coughs. The

urethrocyctometry was performed in lithotomy position. In the cystometry a bladder filling of 500 ml was reached. The urethral profilometry, maximal urethral closure pressure, urinary bladder pressure during filling and the pressures during exertion were registered.

4.7 Statistical analysis

In Study I the differences between groups were tested by using Pearson's chi-square with categorical and one-way analysis of variance (ANOVA) with continuous variables. Spearman´s rank correlation test was used to analyze the correlation between WIS and the three-choice assessment. The associations between the explanatory variables and the severity of symptoms were estimated by a logistic regression model, and the results are presented as odds ratios (OR) with 95% confidence intervals (CI). The severity of the AI symptoms was divided into two groups (no symptoms or mild/severe symptoms) and this was used as an outcome variable. In addition to univariate analysis, models were adjusted for variables clinically valid such as age, body mass index (BMI), parity and mode of delivery. P-value < 0.05 was considered statistically significant.

In Study II the four treatment groups were compared at baseline, at two, and at four weeks. Statistical significance was tested by using chi-square or Fisher's exact test with categorical and one-way ANOVA with continuous variables. The groups and the time points within the groups were compared using ANOVA for repeated measures. Study III was a pilot study testing a novel technique and the patient number was too low to conduct any statistical comparisons. In Study IV a non-parametric Mann-Whitney U test was used for the non-normally distributed parameters of CyQuant measurements. In Studies I and II, the data analysis was performed using IBM SPSS 22 software (Chicago, IL, USA). The version IBM SPSS 23 was used in Study IV.

5 RESULTS

5.1 AI symptoms after primary repair (Study I)

The annual incidence of OASI was 0.7%-1.1% during the study period (2007-2013). Most of the OASI patients were primiparas (76.6%) and 39.8% of the deliveries were vacuum extraction vaginal deliveries. Most of the obstetric tears were stage 3a (44.8%) while only six patients (1.9%) had stage four injuries.

5.1.1 AI symptoms at six months

According to the three-choice assessment, two thirds (64%, n=198) of the patients had no symptoms of AI at six months after OASI repair. On the other hand, 27% (n=85) had AI symptoms and 9% (n=27) of the women wanted further treatment for severe symptoms of AI.

5.1.2 Risk factors for persistent AI symptoms

Advancing maternal age, long (≥45 min) duration of the second stage of labor, occipitoposterior presentation of the fetus, instrumental vaginal delivery, more severe types of injury and a hospital stay exceeding four days were associated with poorer outcomes according to the univariate logistic regression analysis at six

months. In the multivariate model only instrumental vaginal delivery, more severe types of injury and maternal age persisted as associated factors for AI at six months. The operative techniques used or the speciality or experience of the operating doctor (resident gynecologist, specialist in gynecology or colorectal surgeon) did not affect the outcome.

5.1.3 The correlation between WIS and the three-choice assessment

WIS was available for 227 patients (70.1% of the study population); 25% of the patients had WIS 0, referring to no AI symptoms at all. Of the asymptomatic patients, 99.2% (all except one) had WIS 4 or less (Table 8). With mild symptoms, most patients (69.6%) reported WIS between two and three. On the other hand, 81.5% of patients with severe symptoms had WIS 5 or more. The correlation between WIS and the three-choice assessment was good according to the Spearman´s rho correlation assessment (Spearman´s rho 0.82).

Table 8. Number of patients having no/mild/severe symptoms and the Wexner Incontinence Score (WIS).

	No symptoms (%)	**Mild symptoms (%)**	**Severe symptoms (%)**	**Total (%)**
WIS 0-4	120 (99.2)	68 (86.1)	5 (18.5)	193 (85)
WIS 5-14	1 (0.8)	11 (13.9)	22 (81.5)	34 (15)
Total	121 (100)	79 (100)	27 (100)	227 (100)

5.1.4 The delivery mode in the subsequent pregnancy

As of the end of 2014, 106 women of our study population (32.9% of 322) had a new pregnancy and delivery in our hospital. Table 9 shows how the intended and actual modes of delivery were distributed according to the perceived AI symptoms (unpublished data). According to the data, patients with no AI symptoms at 6 months after anal sphincter primary repair were more likely to have an intended vaginal delivery in their subsequent pregnancy while 70% of the women with severe symptoms delivered by elective cesarean section.

Table 9. The proportion of cesarean section and intended vaginal delivery in the subsequent pregnancy grouped by reported AI symptoms at six months after OASI primary repair.

	no symptoms, n= 64 (%)	**mild symptoms, n=32 (%)**	**severe symptoms, n=10 (%)**	**total n=106 (%)**	**p value**
Elective cesarean	21 (32.8)	14 (43.8)	7 (70.0)	42 (39.6)	p=0.07
Intended vaginal	43 (67.2)	18 (56.3)	3 (30.0)	64 (60.4)	
Emergency cesarean	3 (4.7)	0	0	3 (2.8)	

5.1.5 Patients in need of alternative treatment methods

After the six month control, three out of 27 severely symptomatic patients had a secondary sphincter repair operation. Only one of the patients regained anal continence after the operation while two remained incontinent. Most of the remaining 24 patients received extended pelvic floor physiotherapy.

5.2 Adipose stem cell injection therapy in a rat model of AI (Study II)

5.2.1 Functional results after injection therapy

The function of the anal sphincter was tested by anorectal manometry (ARM) as described in Study II. At baseline, the anal pressure was similar in all four groups (Table 10). The functional results (resting median pressure; peak contraction pressure and median contraction pressure during spontaneous contractions) were significantly higher in both hASC-treatment groups compared to the controls without cells at 2 and at 4 weeks. On the other hand, there was no difference in functional results between the two stem cells carriers, 0.9% NaCl and Bulkamid® in the treatment groups, or between the two control groups without cells.

Table 10. The functional results of anorectal manometry at baseline, 2 and 4 weeks in four different treatment groups showed better results in both hASC-treatment groups compared with the controls.

	0.9% NaCl + hASC*	Bulkamid® + hASC**	0.9% NaCl***	Bulkamid®****	p value
Baseline mesurements (mean mmHg; SD)					
Resting median	9.1; 3.4	8.4; 2.8	7.8; 2.4	8.2; 2.3	0.640
Peak contraction	97.5; 25.2	88.5; 29.6	81.3; 22.1	85.0; 23.3	0.349
Contraction median	76.7; 27.0	71.6; 29.2	60.4; 13.8	66.1; 22.3	0.299
Measurements at 2 weeks (mean mmHg; SD)					
Resting median	9.3; 2.6	9.6; 2.1	4.9; 3.2	5.2; 1.6	<0.000
Peak contraction	74.6; 16.0	74.0; 13.6	44.6; 20.6	47.8; 14.3	<0.000
Contraction median	57.5; 12.5	54.4; 11.0	34.1; 17.4	34.3; 12.8	<0.000
Measurements at 4 weeks (mean mmHg; SD)					
Resting median	10.1; 3.0	9.2; 2.3	5.4; 2.5	6.1; 3.4	=0.005
Peak contraction	79.5; 16.4	76.2; 21.3	52.4; 27.2	46.6; 26.7	=0.014
Contraction median	59.5; 10.3	54.8; 12.6	39.2; 18.7	35.1; 18.1	=0.007

*n=14 at 0/2 wk, n=8 at 4wk; **15=0/2 wk, n=8 at 4wk; ***n=15 at 0/2 wk, n=8 at 4wk; ****n=14 at 0/2wk, n=9 at 4wk

5.2.2 Cell characterization and viability in Bulkamid®

The phenotype of the hASCs was assessed at passage 7 using flow cytometry. The mesenchymal origin of the cells was confirmed (Figure 9). The hASCs remained viable in polyacrylamide hydrogel after 3 hours of incubation (Figure 6).

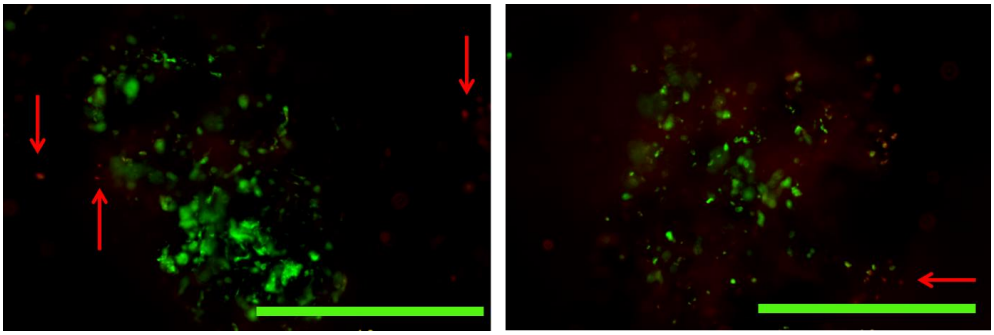

Figure 6. The hASCs remained viable in Bulkamid®. The green fluorescent color represents live cells; red (arrow) shows the dead cells. Scale bar 1000µm.

5.2.3 Histology of the anal sphincter after injection therapy

The Bulkamid® gel was well integrated in the tissue. As explained in the original publication, more inflammation was found in the hASC-groups, especially in the 0.9% NaCl + hASC group. In the histologic assessment, we found that there were no hASCs detectable in any of the samples by the histological and immunohistochemical staining methods. In addition, it was difficult to evaluate the continuity of the muscle layer in the anal sphincter. The cells were labeled with

magnetizing iron particles (PMP-50), but in histology the detected iron was considered to be in the rat macrophages according to the Perls Prussian blue and CD68 staining.

5.2.4 Micro-computed tomography

The μCT was used to visualize the anal sphincter tissue sample area and the injected material and to confirm the continuity of anal sphincter (Figure 7). The continuity analysis according to the histological view was compared to the μCT analysis and there was total agreement in 76% of the samples. Unfortunately, the μCT was unable to confirm the presence of the PMP-50 particles. Additionally, we were not able to reliably assess the sphincter morphology or thickness due to the lack of suitable mathematic models.

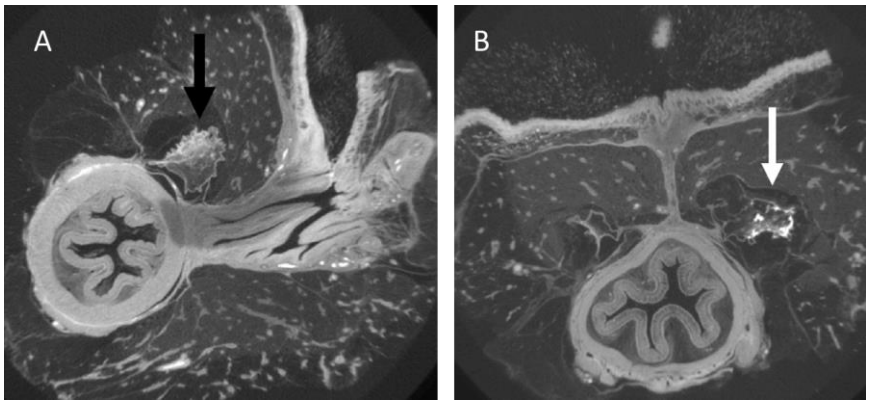

Figure 7. Examples of μCT in order to visualize the magnetizable PMP-50 iron particles. A: Bulkamid®+hASC at 4 weeks; B: Bulkamid® only at 2 weeks. Black arrow = Bulkamid®+hASC-injection. White arrow = Bulkamid®-injection.

5.3 Treatment of women suffering from SUI by autologous ASC+collagen-injections (Study III)

5.3.1 Objective and subjective results

The primary outcome measure was the cough test, which was positive preoperatively in all of the five patients. After six months the cough test was negative for two patients and at 12 months for three patients. Three out of five patients were not satisfied with the cure and wanted to have a mid-urethral sling operation for their SUI. There was no change in urodynamic parameters (urethral profilometry, bladder pressure during filling and exertion) or the residual volume after the injection therapy in any of the patients. According to the questionnaires (UISS, IIQ-7, UDI-6 and VAS) there was subjective improvement of the SUI symptoms with all five patients.

5.3.2 Cell surface marker profile, differentiation potential and viability in Contigen®

The FACS analysis of the surface markers confirmed the mesenchymal origin with positive (≥95%) markers of CD73, CD90 and CD 105 and negative (≤2%) of HLA-DR (Figure 9). Minor expression of markers CD19, CD34, CD45 was detected. The ASCs showed differentiation potential towards adipose, myogenic, chondrogenic and ostogenic cell lineages. The L/D analysis of the ASCs in collagen showed good viability (Figure 8).

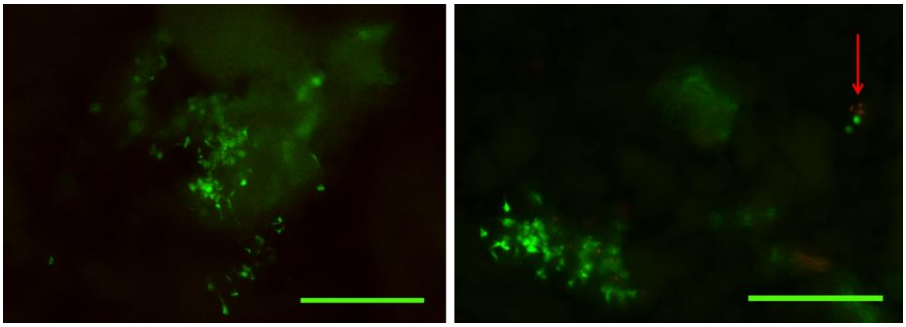

Figure 8. The L/D analysis of the ASCs in collagen. The green fluorescent color represents the live cells, red (arrow) the dead cells. Scale bar 500μm.

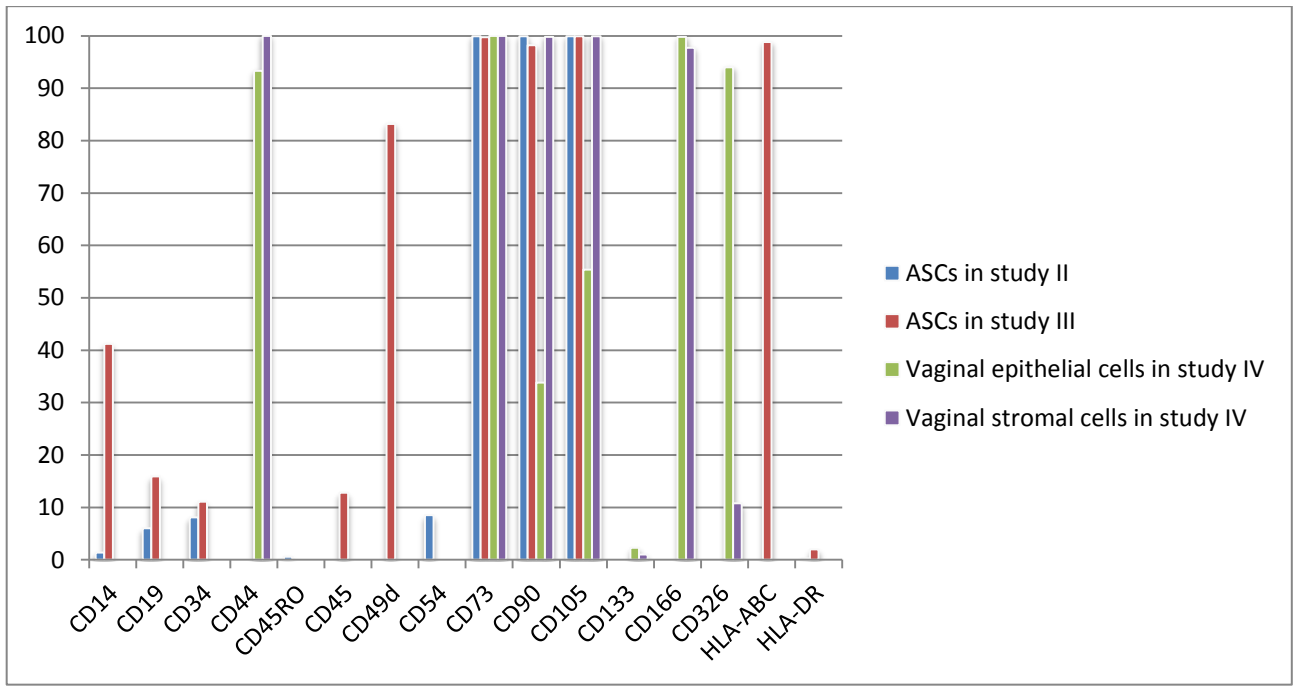

Figure 9. Cell characterization in studies II, III and IV. The surface markers (mean in studies III (n=5) and IV (n=3)) according to flow cytometry. CD=cluster of differentiation.

5.4 Tissue engineering utilizing biodegradable scaffold and vaginal epithelial cells and stromal cells (Study IV)

5.4.1 The biomechanical properties of the scPLSL-scaffold

The average porosity of the PLCL-scaffold was 65 ± 4% and the average pore size 350 ± 150μm measured by the μCT imaging. The tensile properties showed that the elastic modulus was 2.4 ± 0.3 MPa and the material did not break during the tensile test but rather returned to its original dimensions after the straining load was released.

5.4.2 Cell characterization

The flow cytometric analysis of the vaginal epithelial cells and fibroblasts showed that the cell populations of the three different donors were relatively homogenous. The epithelial cells expressed strongly the markers CD44, CD73, CD90 and CD166. There was moderate (20-60%) expression of markers CD90 and CD105. The hematopoietic or endothelial markers showed low expression. The vaginal stromal cells expressed strongly markers CD44, CD73 and CD166 and CD326. There was moderate expression (20-60%) for markers CD90, CD105 and very low expression for CD133 (Figure 9).

5.4.3 Cell attachment, viability and proliferation on the PLCL scaffold

According to the L/D analysis, the viability of the both cell types on the scPLCL scaffold was good in epithelial and stromal cell cultures at all the three time points (1d, 7d, 14d). In the co-culture, the epithelial cells grew better due to the more favorable environment of the Epilife® culture medium. The cells were homogenously spread on the surface of the material; especially the epithelial cells favored the pores of the scPLCL-scaffold (Figure 10). Also, the SEM analysis showed distribution of both epithelial and stromal cells on the scaffold surface.

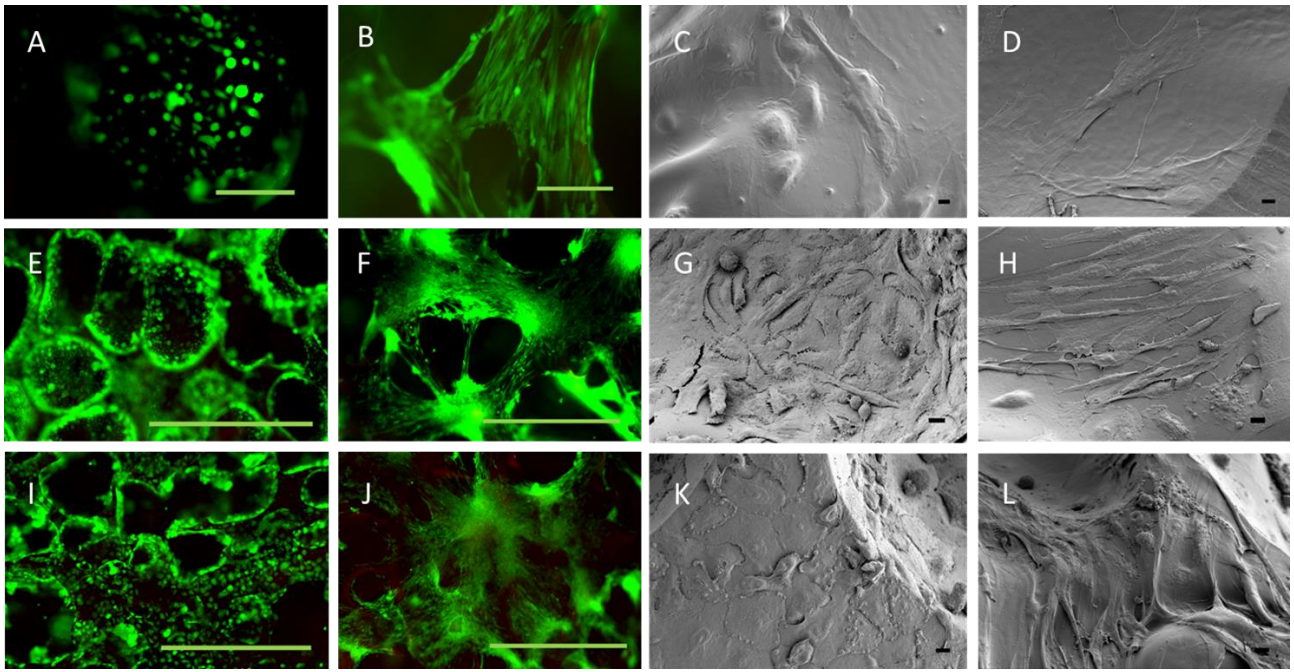

Figure 10. The epithelial and stromal cells on the PLCL scaffold. A,B,E,F,I,J: L/D-images of the epithelial and stromal cell cultures at different time points. C,D,G,H,K,L: SEM-images of the co-cultures at 1, 7, 14d. A: Epithelial cell culture at 1d, scale bar 200µm. B: Stromal cell culture at 1d, scale bar 200µm. C: Epithelial cells in co-culture, 1d. Scale bar 10 µm. D: Stromal cells in co-culture, 1+5d. Scale bar 10 µ. E: Epithelial cell culture at 7d. Scale bar 1000µm. F: Stromal cell culture at 7d. Scale bar 1000µm. G: Epithelial cells in co-culture, 7d. Scale bar 10µm. H: Stromal cells in co-culture, 7+5d. Scale bar 10µm. I: Epithelial cell culture, 14d. Scale bar 1000µm. J: Stromal cell culture at 14d. Scale bar 1000µm. K: epithelial cells in co-culture at 14d. Scale bar 10µm. L: Stromal cells in co-culture at 14+5d. Scale bar 10µm.

6 DISCUSSION

In this thesis we wanted to assess the possible role of tissue engineering techniques in the treatment of pelvic floor disorders. So far these techniques are experimental, but women's attitudes towards tissue engineering techniques are mainly positive; 84% of women are willing to consider autologous cell-based methods for obstetric injury-induced UI or AI (Wright et al. 2016).

If surgery rates remain at the current level, there will be more women needing surgery for UI and POP in the future (Wu et al. 2011b). Although the current operative treatment methods are suitable for most patients with pelvic floor disorders, in the future there may be an increasing need for alternative tissue engineering–based treatment methods also. Not all patients achieve satisfactory results with the current operative methods, and women's expectations and use of QoL-criteria are placing demands on the treatment methods.

6.1 Novel treatment methods for AI are needed

It is evident that pregnancy and childbirth increases the risk for pelvic floor disorders in women (Lipschuetz et al. 2015). In our first study we aimed to recognize risk factors for persistent AI symptoms and to assess the need for alternative treatment methods. In order to avoid OASI it is important to recognize the risk factors for OASI: primiparity, vacuum delivery, occipitoposterior position and fetal weight >4000g (de Leeuw et al. 2001; Christianson et al. 2003; Gottvall et al. 2007; Eskandar and Shet 2009; Jango et al. 2014). Moreover, recognition of the risk factors for persistent symptoms of AI is important to optimize the treatment of OASI. In our study, advancing maternal age, the severity of the injury and

instrumental vaginal delivery were independent risk factors for severe symptoms of AI at six months after primary anal sphincter repair. Our protocol with OASI primary repair seems effective in restoring the pelvic floor and in preventing short term complications.

The proper diagnosis of anal sphincter damage is essential to adequately treat the rupture. Sphincter injury can be missed because of tissue edema and bleeding. Education of the staff (midwives, trainees, residents, consultants) is crucial for proper recognition of sphincter ruptures. There are also reports of occult anal sphincter ruptures, so a normal perineum does not exclude underlying sphincter damage (Frudinger et al. 1997), although true occult injuries are rare. The more experienced the clinician, the less likely that the injury is missed (Andrews et al. 2006a).

It has been suggested that the results of primary sphincter repair can be improved if the operation is performed by a colorectal surgeon (Cook et al. 1999; McNicol et al. 2010). On the other hand, there is evidence that trainees in obstetrics have better understanding of the classification and the consultant obstetricians have the largest experience in OASI surgery (Fernando et al. 2002). In our study, there was no difference in results according to the specialization of the operating doctor (resident, specialist in obstetrics and gynecology, specialist colorectal surgeon). However, the fact that residents mainly operated on milder ruptures and that the statistical power was not calculated beforehand must be kept in mind when interpreting the results.

There is also a need for practical methods to find those women who will need further treatment to better target health care resources. Not all women need a routine follow up after OASI primary repair. In our study, we found a simple three-choice assessment, together with WIS, useful in recognizing women with severe AI symptoms needing treatment. Different kinds of questionnaires have been used before to assess the AI symptoms. The three-choice assessment gives more information than a VAS-scale (Paka et al. 2016) and with WIS more specific information can be gathered. The three-choice assessment needs to be validated and translated in English for wider use. It is difficult to compare symptoms and different treatment methods in different studies, because the outcome measures vary widely. Standardization of the evaluation methods is needed.

At six months, AI symptoms were present in 9% of women with OASI primary repair in our study. This result parallels a British study (Ramalingam and Monga 2013). In an older cohort study from Finland, the results of OASI primary repair were considerably worse, as even 61% of women suffered from AI after 15 months follow up (median, range 2-144 months) (Pinta et al. 2004). Also, Farrell et al. found in their randomized trial comparing operative techniques that 39% (end-to-end) to 61% (overlapping) of primiparas suffered from flatal incontinence at six months. At three years the flatal incontinence still bothered 39% and 43% of women, respectively. The difference between the two operative techniques disappeared at the two year time point. (Farrell et al. 2012) OASI is not, however, the only risk factor for AI. Regardless of the delivery mode, AI occurs in large numbers of women 30 years after delivery; flatal incontinence up to 58.6% in the sphincter injury group vs. 30.3% in the episiotomy only group and 15.2% in the cesarean section group. The figures for bothersome FI were 27.6%, 25.8% and 15.2%, respectively. (Nygaard et al. 1997)

Secondary sphincteroplasty used to be the gold standard for treating AI in patients with nonfunctioning and scarred anal sphincters. However, in our material only one of the three patients with secondary sphincteroplasty regained anal continence after the operation. Thus, new treatment methods should be sought. Nowadays the neuromodulation technique, especially SNM, is considered to be an effective treatment option (Thin et al. 2015). Bulking agents have been tested for AI in a few small clinical trials, but the quality of the studies is insufficient to draw further conclusions about the efficacy (Maeda et al. 2013). The use of bulking agents in AI has also been criticized. Anal and urethral continence mechanisms differ, especially in view of the relative size of the anus and urethra. It has been suggested that although a non-migrating substance might mechanically occlude the urethra, it might not be the case in the anus, which has a much wider lumen that already accommodates the volume of anal cushions or hemorrhoids. Any effect of injections must therefore be via a different mechanism than from direct occlusion (Norton 2011). Small trials with stem cells have also been conducted, but the patient groups have been heterogeneous and further studies are needed (Frudinger et al. 2010; Frudinger et al. 2015; Romaniszyn et al. 2015; Boyer et al. 2017; Sarveazad et al. 2017).

6.2 Human adipose stem cell-injection therapy enhances anal sphincter contraction in rats

The purpose of our second study was to develop an alternative treatment method for AI resulting from anal sphincter injury in an animal model. We used a rat model to mimic an acute anal sphincter injury resembling a fourth degree injury in humans. Two different carriers, 0.9% NaCl and a synthetic polyacrylamide hydrogel Bulkamid® were used to enhance the healing of the anal sphincter. The functional tests were conducted by *in vivo* ARM.

There is some variation in anal pressures between different studies (Kajbafzadeh et al. 2010; Oh et al. 2015; Lane et al. 2013; Salcedo et al. 2014). In humans the resting pressure is considered to reflect the function of the IAS as the maximal contraction is generated by EAS. For animals, on a smaller scale and with involuntary spontaneous contractions, there is no evidence that the mechanism is the same, although there is also no evidence to the contrary. The ARM measurements in our study showed that the functional properties of the treated anal sphincter were significantly better in the hASC-groups compared to the controls. Other research groups have produced similar results in stem cell treatment. In a study of Salcedo et al. the MSC-therapy improved the anal sphincter function in the sphincterotomy group regardless of the cells administration route. In the pudendal nerve crush group there was no benefit of the stem cell therapy reflecting a different injury mechanism (Salcedo et al. 2013). The same research group found that both serial i.v. infusion and i.m. injections of MSCs after partial sphincter excision resulted in increased anal pressures. The MSC-treated groups showed less scarring and fibrosis in the anal sphincter histology than the saline-treated groups (Salcedo et al. 2014). On the other hand, Pathi et al. found no benefit with i.v. injection of the MSCs, while local injection enhanced the rat anal sphincter function (Pathi et al. 2012). Lane et al. showed that after injecting rat muscle stem cells in the injured anal sphincter the anal pressure returned to preoperative level in stem cell treatment group but not in saline controls (Lane et al. 2013).

The polyacrylamide hydrogel was a suitable carrier for the stem cells. In our animal model there was no advantage of Bulkamid® over the 0.9% NaCl-controls, which refers to the fact that the bulking effect was not present. There was less

inflammation in the hASC+Bulkamid®-group compared to the hASC+saline-treatment. It is not clear, however, what grade of inflammation is desirable for tissue to heal. Bisson et al. showed that an injection of myoblasts on the opposite site of the anal sphincter injury did not result in better function and they concluded that a certain amount of inflammation is required for the stem cells to have an effect (Bisson et al. 2015). It seems that the Bulkamid® gel inhibits immunological reactions or inflammation elicited by the human ASCs in a rat model, but, on the other hand, it did not inhibit the positive effect of hASCs.

One of the major contributors of cell therapy is the promotion of tissue remodeling and regeneration by induction of tissue vascularization, immunomodulatory effects and the secretion of growth factors (Dimarino et al. 2013). There is evidence that ASCs are producing high levels of VEGF and therefore stimulate the formation of capillary vessels (De Francesco et al. 2009). MSCs have also been shown to be capable of modulating the immune response in different tissues (Ghannam et al. 2010; Nauta and Fibbe 2007). The secretion of specific soluble growth factors (e.g. fibroblast growth factor, insulin-like growth factor, transforming growth factor) is considered to be one of the mechanisms of action at the tissue level (Rehman et al. 2004; Salgado et al. 2010). In our study, there was no difference in anal sphincter muscle continuity between the four treatment groups, which implies that perhaps the mechanism of hASCs in rat anal sphincter rehabilitation is based on paracrine effect rather than muscle differentiation.

We were not able to find PMP-50-iron particles in the rat anal sphincter samples either in μCT or histological evaluations. Different methods of labeling the injected cells have been in use in previous studies. Graig et al. transplanted green fluorescent protein (GFP)-labeled myoblasts extracted from the rat skeletal muscle into the intact anal sphincter muscle using electromyographic guidance. The sphincter was surgically extracted after ten days and histologically analyzed. The GFP-labeled cells were present in the sphincter tissue (Craig et al. 2010). The same group continued MSC-treatment with Sprague-Dawley rats with myogenic stem cell injection after proctoepisiotomy and found no difference in sphincter morphology in histologic tissue analysis. Furthermore, the nuclear staining did not demonstrate a significant difference in size, appearance or number of nuclei between treatment and control groups (Lane et al. 2013). Salcedo et al. found no GFP-positive stem cells after i.m. or i.v. administration of MSCs in rat anal

sphincter in the immunofluorescence analysis (Salcedo et al. 2013; Salcedo et al. 2014).

Our model resembles an acute anal sphincter trauma that is immediately corrected in laboratory circumstances without the nerve distension and edema that often complicates the obstetric ruptures. The ASCs we used were retrieved from a single donor. There might be differences in different donors that could affect the properties of the injected stem cells (Choudhery et al. 2014; Serena et al. 2016). In clinical practice, human AI is usually a chronic condition with fibrous scarring and denervation of the sphincter, and age, obesity, pelvic floor dysfunction and dietary matters have an impact on the symptoms. More studies are needed in order to find an ideal technique, carrier and timing for the tissue engineering techniques.

6.3 Autologous ASCs in transurethral injection treatment of UI may restore continence in some patients

In our third study we studied transurethral injections of autologous ASCs combined with collagen gel to treat SUI. Urethral bulking agents have been used for SUI for years with limited results. Numerous different agents have been tested, some with serious late complications and side effects (Dmochowski and Appell 2000). On the other hand, Mohr et al. treated 500 patients with different bulking agents with a negative two hour in-office pad test in 73.2% of the patients, and improvement of VAS for incontinence symptoms (Mohr et al. 2013). The advantage of bulking agent treatment is that it is a fairly simple outpatient procedure with relatively low cost and rapid recovery (Kirchin et al. 2012).

Mid-urethral slings are at the moment the first-line surgical treatments for SUI. Reported cure rates rise even as high as 90% (Nilsson et al. 2008). The results of the operation seem to be good, even after the first ten operations performed, but experience of the surgeon can significantly improve the results and minimize complications (Serati et al. 2015; Montera et al. 2016). The results after both

retropubic and transobturator techniques are similar and over 80% of women are continent after five years (Laurikainen et al. 2014). However, 10-20% of patients are in need of alternative treatment methods.

Especially in the Western world, obesity has become one of the largest health problems and one of the most common procedures in plastic surgery is abdominoplasty. Therefore, adipose tissue forms an enormous source of possibly functional material, e.g. for tissue engineering. Adipose tissue is a composition of adipocytes, endothelial cells, endothelial progenitor cells, pericytes and SVF (Morcos et al. 2015). Both in humans and in rodents there is a homeostatic mechanism that maintains the balance between fully differentiated adipose cells and the undifferentiated stem cells in adipose tissue (Gimble et al. 2007). Human lipoaspirate contains multipotent cells that show differentiation in vitro into adipogenic, chondrogenic, myogenic and osteogenic cells in the presence of lineage-specific induction factors (Zuk et al. 2001). ASCs have proved to be an easily accessible source of cells (Gimble et al. 2007). Compared to other cell sources (MDSCs, BMSCs) the ASCs are less invasive to obtain and do not disturb the normal function of an organ. In our Study III, all the patients had a sufficient amount of subcutaneous fat for adipose tissue retrieval in local anesthesia. We were able to successfully culture the cells, grow them in collagen and perform the transurethral injections without complications.

Several culture media have been tested to induce ASCs and BMSCs differentiation towards skeletal muscle (Lo Furno et al. 2016). ASCs have shown high proliferation rates and expression for myogenic markers such as skeletal muscle actin-α 1, myosin heavy chain 1 and 8 and desmin (Stern-Straeter et al. 2014). When culturing ASCs together with murine C2C12 myoblasts, the ASCs have proven to fuse and express human sarcomeric proteins (Dugan et al. 2014). ASCs have proven to differentiate towards smooth muscle phenotype both in basal growth media and especially with induction supplements. In our study the ASCs showed positive expression for myogenic markers, thus demonstrating a myogenic differentiation potential. On the other hand, the amount of muscular tissue in female urethra could not be measured to see if the myogenic differentiation resulted in actual muscle tissue growth *in vivo*. Most likely the effect of MSCs is a paracrine effect: the injected ASCs enhance the growth and vascularization of the host tissue and even improve the nerve function.

According to a recent review there are no other clinical studies utilizing ASCs in treatment of female SUI (Vinarov et al. 2017). In our pilot study three out of five patients had a negative cough stress test at 12 months and two of them were satisfied with the cure. The cough test is a useful method in diagnosing SUI and assessing the result of the operative treatment. However, the cough tests are not fully standardized. Although results of mid-urethral slings are superior when compared to the results of our small study with heterogenous patient material, the advantage of this technique is that an operation with synthetic non-degradable material is avoided. Furthermore, our technique seems to be a safe alternative, which is important when dealing with novel techniques. It is possible that there is a learning curve for this procedure which would impact improvement of the results in larger series.

6.4 PLCL-scaffold is a promising biomaterial for vaginal tissue engineering

Several biomaterials have been tested for epithelial reconstruction of vaginal agenesis, serious mesh complications or gynecological cancer surgery defects or reconstruction of transgender operations, but so far the ideal material for reconstruction has not been found.

For vaginal reconstruction the biomaterial should be elastic, flexible, biocompatible and suturable. There are increasing numbers of reports on the effect of age, hormonal status and delivery on the biomechanical properties of the vaginal tissue (Ulrich et al. 2014; Urbankova et al. 2018). Yet, the ideal properties are to be determined. The mechanical properties of the porous scPLCL-scaffold in our study were tested by strain and stress test and the elastic modulus showed that the material is resistant for tensile strain and that it returned to its original shape after the strain was released. This is an important property when developing biomaterials that have to resist physical activities, e.g. in physical exercise. Supercritical carbon dioxide (CO_2) foaming is a method for fabricating 3D scaffold in order to avoid any harmful solvents in fabrications process (Kim et al. 2013). According to our

results, the tensile and elastic properties of scPLCL seem to be suitable for vaginal tissue engineering.

Natural and synthetic scaffolds have been tested in animal models and a there are a few successful clinical trials to replace urinary bladder, urethra or vagina in humans (Atala 2006; Raya-Rivera et al. 2011; Raya-Rivera et al. 2014). A nonimmunogenic synthetic graft could hopefully also minimize the risk of malignant transformation or the unpleasant side-effects (mucous excretion, odor, hair growth, dryness, neovaginal prolapse) of the natural graft materials that are currently in use (Hensle et al. 2006; El-Sayed et al. 2007; Cao et al. 2013; Choussein et al. 2017). There are, however, several safety issues including the risk of graft ischemia, fibrous contraction and perforation that have hindered the clinical use of the advanced technologies (Alberti 2016). Since the first case reports, very few trials have been conducted and published about further development of tissue engineering in successful development of urethral, bladder or vaginal neo-organs. Further preclinical studies on the usability and safety of the scPLCL are needed before clinical translations take place.

According to our study, the epithelial cells and stromal cells derived from vaginal tissue can be separated and seeded on the scPLCL scaffold. The flow cytometry showed that both epithelial and stromal cells were present in the co-culture despite the more favorable culture media towards the epithelial cell growth. The 3D porous structure and the pore size were also favorable, especially for the epithelial cells. In the SEM imaging the cells were attached and evenly distributed on the scPLCL surface, which is an important finding for the further development of the biomaterial. The separate cultures of epithelial and stromal cells grew well and the cells maintained their phenotype. To the best of our knowledge the vaginal epithelial and stromal cells have not been characterized before. Thus, our study provides a surface marker profile for vaginal epithelial and stromal cells.

6.5 Methodological considerations

Study I was a retrospective register-based study representing the clinical practice in a decidedly large hospital. The patient material was not selected and therefore it represents a general obstetric population and the results are comparable to many clinical settings. Also, the study reflects the situation in a teaching hospital where education and development are important issues and the number of attending doctors is large. The data was collected from hospital register which is a reliable resource of information. Not all patients returned the actual form; therefore some of the WIS-data was not available.

In Study II we studied hASC-injections combined with a carrier in a rat model. The rats were normal Sprague-Dawley rats without immunosuppression and the hASCs were collected from a single donor without donor variability. The rat anesthesia, the technique for rat ARM, anal sphincter cutting and repair and the injection technique were carefully tested beforehand in a pilot study with 24 rats. However, the rat anal sphincter is small and the Bulkamid® gel was not ideal for miniature surgical conditions. The injection technique was new with only a few reference studies. For clinical use those difficulties can probably be solved due to the larger scale in human anatomy. The rats were examined by ARM before and after the treatment and the treatment protocol was consistent since all treatments were conducted by the same researcher. To ensure the objectivity of the ARM data analysis, the functional results were revised by a blinded researcher. The analysis for histology and immunohistochemistry were conducted in a laboratory familiar with this type of sample handling. We found no previous references for the muscle continuity assessment in μCT.

There are several animal models that have been tested for methods of reconstruction of the pelvic floor. However, the animal models are not ideal since the effect of gravity is different on quadrupeds than in humans in vertical position and the effect of delivery is somewhat different. Rats are the most commonly used animals in incontinence models. There are many similarities between human and rat anatomy and physiology. However, the muscular tissue is different since rats have more fast-twitch muscle fibers than humans (Abramowitch et al. 2009; Poortmans and Wyndaele 1998). We could not verify the presence of the PMP-50-

labeled hASCs in rat tissue probably due to rat immune defense reaction. Our AI-model is a model of an acute trauma. In humans, AI is often a result of a poorly functioning fibrous sphincter and models for chronic injury need to be developed. Furthermore, the ASC-injection therapy for human AI treatment has to be tested in clinical trials.

Study III was the first clinical study utilizing hASCs in treatment for UI. All treatments and controls were done by one researcher, which may have influenced the patients' subjective assessment of the efficacy of the treatment. The number of patients was small and no comparisons with the standard treatment methods were made. The patients were carefully examined and the data is reliable. There was no funding for an extension of the study and because of the high cost of the treatment and the unavailability of the Contigen® gel, the study was not continued.

In Study IV the method of handling the vaginal tissue was new. The isolation of the vaginal epithelial cells from the stromal cells was performed several times to achieve the best results. For the co-culture, the growth medium favored the epithelial cells which compromised the growth of the stromal cells. Further studies are required to assess the suitability of the scPLCL-scaffold *in vivo* animal models before clinical translations.

6.6 Ethical considerations

Our studies were conducted by following the principles of the WMA Helsinki declaration and the Finnish legislation.

In Study I the most important ethical consideration was interpreting the data into clinical relevance. For Study II the 3R principle of animal testing was followed. Replacement of the animals by *in vitro* cell lines was not possible since functional assessment and tissue reactions to the treatment could not be tested without live animals. Reduction of the amount of animals was done by carefully planning the trial to achieve statistical power. Refinement was done by choosing the smallest

possible animal according to previous studies, by ensuring the appropriate facilities, adequate pain relief and other medication as well as care-giving personnel to ensure the animals' well-being.

Human integrity is especially important when offering patients purely experimental treatments, such as the SUI injection treatment with autologous stem cells in Study III. Informed consent has to be based on carefully communicated and understandable information about the benefits and risks of the experimental treatment. For stem cell research, one of the concerns is the possible malignant transformation of the multipotent cells. Before starting the study careful review of the literature was performed and no signs of adverse reactions were reported.

The tissue samples for Study IV were acquired from transgender patients that had an elective operation for vaginectomy to remove the vaginal epithelium. Informed consent was acquired and the study did not require any extra visits from the patient. Anonymity is especially important in sensitive issues like transgender treatment.

Study I was a register based study and it was performed under the approval of the Pirkanmaa Hospital District Science Center (R13568). Study II was approved by Regional State Administrative Agency (ESAVI-2828-04 10 07-2015) for the use of animals in the testing. Pirkanmaa Hospital District Ethical Committee (R15161) has given supportive statement for the use of hASCs. Study III was based on patient informed consent and had a supportive statement by Pirkanmaa Hospital District Ethical Committee (R09179). Study IV had a supportive statement by the Pirkanmaa Hospital District ethical committee (R15051).

6.7 Future perspectives

Tissue engineering holds great promise for the treatment of functional disorders. Earlier studies have concentrated mainly on the differentiation of stem cells towards different cell types. Further studies have emphasized the paracrine effects of especially the MSCs and the improvement of functional parameters instead of histologically confirmed differentiation, e.g. muscle formation. The possible delivery methods are direct transplantation or local injection of the cells at the site of injury or intravenous or sometimes intra-arterial infusion, although results are contradictory (Pathi et al. 2012; Salcedo et al. 2014). The mechanism via which stem cells might migrate to the injury site is still somewhat unknown. Bisson et al. stated that some amount of acute trauma and inflammation is needed to achieve a favorable effect from stem cell transplantation.

For AI, there is a need for a minimally-invasive and safe method to cure the AI symptoms, both after a failed OASI primary repair in younger women and later in life when pelvic floor disorders become more common. The establishment of the ideal method and carrier material will need further studies. The different mechanisms of acute and chronic trauma and especially the effect of the ASC-treatment on chronic conditions are of utmost importance. Additionally, the possibility to use allogenic stem cells or freshly isolated SVF will reduce the cost and might facilitate clinical use.

Even though the long term results with mid-urethral slings are good and injection treatments with traditional bulking agents are technically simple procedures, there is a need for alternative treatment options. Our Study II revealed that Bulkamid® might be a suitable carrier for stem cells for injection therapy because of the good handling properties and integration to the tissue with minor foreign body reaction. In the future, the possibility to combine Bulkamid® with ASCs in the treatment in UI needs to be evaluated. Also, a biodegradable scaffold utilizing the rationale of integral theory and the paracrine effect of the stem cells might be a worthwhile option.

PLCL seems to be a potential scaffold for epithelial and stromal cells. Further use with other types of cells, especially stem cells opens new possibilities in the

treatment of pelvic floor disorders. For vaginal agenesis and other congenital disorders there are many possibilities to develop methods that can create new epithelial tissue from small amounts of cells. For transgender reconstructive surgery a tube-like form would promote the possibility to develop a replacement for urethral reconstruction in a phalloplasty operation. Also, the use of stem cells and their potential to enhance the functional properties of the pelvic floor in the treatment of POP is to be further studied. Primary cells are an option in developing new applications for reconstructive surgery. Hung et al. stated in their study that tissue-engineered fascia could be developed from human vaginal fibroblasts combined with collagen *in vitro* and *in vivo* and this might be an alternative treatment for POP in the future (Hung et al. 2010). Further preclinical and clinical studies on the tissue reactions and functional properties of the scPLCL *in vivo* are needed.

An interesting possibility would be the secondary prevention of AI or UI at the time of delivery. That would mean optimal recognition of the risk factors, minimizing the damage to the pelvic floor during delivery and optimizing the healing process, perhaps by injecting stem cells (Callewaert et al. 2017). The clinical use of autologous stem cells is restricted by the long timeframe required for the cell retrieval and culturing. This also produces a significant economic burden with the high laboratory expense. SVF-cells isolated from fat tissue on the other hand can be transferred to the patient within an hour. Allogenic transplantation is limited with the techniques used today by the fact that the immune response might easily deteriorate the positive effect of the foreign stem cells. Xenograft or allogenic cells would lower the cost with the ability to produce larger amounts of commercial cell lines. However, it is still to be determined for which clinical applications autologous and allogenic cells are optimal cell sources. It is also very important that strict GMP-guidelines are followed when culturing and preparing cells for clinical translations (Riis et al. 2015).

7 CONCLUSIONS

The pelvic floor disorders are common and disturbing conditions for many women. The treatment methods today for OASI, AI, UI, POP and congenital vaginal agenesis are quite effective. However, there are still patients who are not helped by conservative or conventional surgical methods and tissue engineering might have something to offer. The main findings and the conclusion were:

1. According to our results, 9% of women have disturbing symptoms of AI at six months. Severe types of injury, advancing maternal age and instrumental vaginal delivery are independent risk factors for more severe AI symptoms. The empirically developed three-choice assessment of AI has a good correlation with the widely used WIS and it might help in targeting health care resources to patients who need further treatment.

2. Injection therapy using hASC together with carrier shows superior short term results in curing iatrogenic anal sphincters in rats, when compared with controls treated by carriers only. Polyacrylamide hydrogel is suitable biomaterial for cell based therapies and seemed to produce less inflammation than 0.9% NaCl as a carrier.

3. Based on the patient material of five women suffering from SUI, periurethral injection therapy of ASC combined with collagen has no short term adverse effects. The efficacy of the ASC-therapy needs to be further evaluated since only three out of five patients benefited from the ASC-injection according to our primary outcome measure, the cough test.

4. The novel scPLCL scaffold is suitable for growing vaginal epithelial and stromal cells. More studies are needed to develop an optimal seeding environment for co-culturing different cell types. The biomechanical properties of scPLCL seem suitable for vaginal tissue engineering based on our *in vitro* studies.

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10 ORIGINAL COMMUNICATIONS

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ORIGINAL ARTICLE

Outcomes of primary anal sphincter repair after obstetric injury and evaluation of a novel three-choice assessment

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Abstract

Background The aim of the present study was to evaluate the subjective outcome of primary repair of obstetric anal sphincter injury (OASIS) at 6 months, the factors associated with the symptoms of anal incontinence (AI), and the role of a simple survey consisting in one question with three answer choices, combined with the Wexner incontinence score for the assessment of this patient population.

Methods A retrospective cohort study was conducted on patients with third- or fourth-degree OASIS operated on between January 2007 and December 2013 inclusive at Tampere University Hospital, Finland. At 6 months, the patients were asked to report their Wexner's score as well as the three-choice assessment regarding AI symptoms. Based on this assessment, the patients were divided into three groups: those, asymptomatic, those with mild symptoms who did not want further treatment and those with severe symptoms who were willing to undergo further evaluation and treatment.

Results There were 325 patients (median age 30 years). A total of 310 patients answered the questionnaire. Of which, one hundred and ninety-eight (63.9%) patients were asymptomatic, 85 (27.4%) had mild AI, and 27 (8.7%) experienced severe symptoms. There was no statistical difference in the results between the two techniques used (overlapping vs. end-to-end), or the stage of specialization of the operating physician. Persistent symptoms were associated with instrumental vaginal delivery (OR 2.12, 95% CI 1.32–3.41), severity of the injury (OR 1.64, 95% CI 1.20–2.25), and increased maternal age (OR 1.07, 95% CI 1.02–1.13). The correlation between the three-choice symptom evaluation and the Wexner score was good (Spearman's rho 0.82).

Conclusions After 6 months, severe symptoms after OASIS repair were present in 9% of women and were more frequent in older women, women with high-degree tears and after instrumental vaginal delivery. A three-choice assessment of AI symptoms correlated well with the Wexner score and might be useful to triage patients who need further evaluation.

Keywords Anal sphincter · Anal incontinence · Fecal incontinence · Obstetric anal sphincter injury

Introduction

Anal incontinence (AI) is defined by involuntary loss of feces or flatus [1]. The most common traumatic cause for AI in women is obstetric anal sphincter injury (OASIS) [2]. The incidence of obstetric third- and fourth-degree anal sphincter rupture varies from approximately 11% [3] worldwide to 0.6–4.2% in Nordic countries [4]. Known risk factors for OASIS are high fetal birth weight, long duration of second stage of delivery, operative delivery, primiparity, and midline episiotomy [5, 6].

AI despite primary OASIS repair has been reported to occur in 61% of patients [7]. The extent of sphincter damage [8], operative vaginal delivery [9], older age, and high body mass index (BMI) [10] are associated with the risk of fecal incontinence after primary repair.

In Finland, extensive and continuous efforts have been made to prevent OASIS and to improve the quality of diagnostics and repair [11].There has also been discussion about the optimal specialization (gynecologist or colorectal surgeon) of the operating physician and surgical team [12].

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Outcomes after OASIS have improved with time, and the symptoms of anal incontinence are reported less frequently than previously [7].

The aim of this study was to examine the subjective outcome of OASIS primary repair surgery and to recognize the factors associated with persistent AI symptoms. We also evaluated the role of a simple three-choice assessment combined with the Wexner incontinence score [13] in patients with OASIS.

Materials and methods

A retrospective cohort study on women with OASIS was conducted between January 2007 and December 2013 inclusive at Tampere University Hospital, Finland, a tertiary care teaching hospital with up to 5400 deliveries per year. The yearly cesarean section rate varied between 14.6% (2012) and 17.7% (2007), and the rate of operative vaginal deliveries from 5.6% (2013) to 8.1% (2008). At our hospital, the perineum is supported to prevent perineal tears in almost all deliveries [11]. According to the classification of OASIS, a third-degree injury involves the anal sphincters; (3a) involves less than 50% of thickness of the external anal sphincter, (3b) more than 50% of thickness of the external anal sphincter, and (3c) both the external and internal anal sphincter. Fourth-degree injury extends to the anorectal mucosa [14]. Our initial analysis was made of patients diagnosed with all types of third-degree injuries as well as fourth-degree obstetric anal sphincter injuries. Recurrent anal sphincter injuries (three cases, recurrence rate 5% of attempted vaginal deliveries) were excluded from the analysis.

At 6 months, the patients were sent a questionnaire about subjective AI symptoms including a Wexner incontinence score sheet and a three-choice assessment form. The Wexner incontinence score contains questions about the frequency and type of incontinence or discomfort (solid stool, liquid stool, flatulence; the use of diapers or pads, lifestyle changes). A score of 0 corresponds with full continence, while a score of 20 corresponds with total incontinence [13]. The empirically developed assessment asking patients which of three answer choices best described their condition is: (1) the patient is satisfied and has no symptoms (=no symptoms); (2) has mild symptoms but does not wish for a doctor's appointment (=mild symptoms); and (3) has severe symptoms and wants to be contacted by a colorectal surgeon (=severe symptoms). This has been used to target the resources for women who want further treatment. The patients who reported severe symptoms were examined by a colorectal surgeon with endoanal ultrasound and anal manometry. A follow-up visit was arranged within 7–14 months of the initial injury.

In order to examine the variables associated with persistent AI among patients diagnosed with third- and fourth-degree obstetric anal injuries, the women were divided into two groups based on the three-choice assessment: those who reported no AI symptoms at 6 months after OASIS ($n=198$), and those who had minor or severe symptoms ($n=112$). The study population is described in Fig. 1. The groups were compared using pre-labor and intrapartum factors.

Statistical analysis

Statistical significance was tested by using Pearson's Chi-square test with categorical and one-way analysis of variance (ANOVA) with continuous variables. Spearman's rank correlation test was used to analyze the correlation between the Wexner incontinence score and the subjective outcome measure. The associations between different explanatory

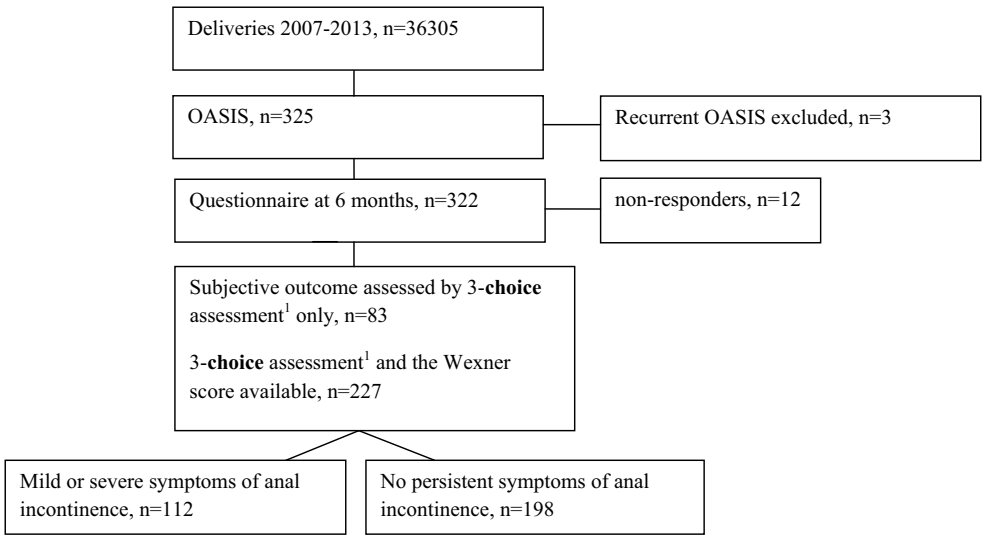

Fig. 1 The patient flow chart

variables (Table 1) and the severity of symptoms were estimated by a logistic regression model, and the results were presented as odds ratios (OR) with 95% confidence intervals (CI). To distinguish between the asymptomatic and symptomatic groups, the severity of the anal incontinence symptoms (0 = no symptoms, 1 = mild/severe symptoms) was used as an outcome variable. In addition to univariate analysis, the models were adjusted for clinically valid variables, such as age, BMI, parity, and mode of delivery. A p value of < 0.05 was considered statistically significant. The data analysis was performed using IBM SPSS 22 software (Chicago, IL, USA).

Results

Three hundred and twenty-five patients were diagnosed with OASIS between the start of 2007 and the end of 2013 from a total of 36,305 deliveries. Primiparas accounted for 42.4% of in the total number of obstetric patients, but 76.6% of those who had OASIS. One hundred and ninety-four patients with OASIS (60.2%) had a spontaneous vaginal delivery (1 breech presentation), whereas 128 (39.8%) had an instrumentally assisted delivery (vacuum: 127, forceps: 1). A mediolateral episiotomy was performed in 188 (58.4%) cases), which differs from the total annual episiotomy rate in the hospital of 20–26% during the study period. The annual incidence of OASIS ranged from 0.69 to 1.10%.

All patients were operated on within 24 h of the delivery by either a resident, specialized gynecologist, or a colorectal surgeon. During their hospital stay, the patients received prophylactic antibiotics and had physiotherapy counseling including perineal inspection, pelvic floor exercise instructions as well as dietary information. The physiotherapist examined the patients and their pelvic floor function 3 months after the delivery.

Most of the patients had stage 3a (44.8%) or 3b (41.6%) tears, 11.6% had 3c, and only six patients (1.9%) had a stage 4 rupture. Sixty-five doctors were involved in the operations either operating or assisting, and the operation volumes per

Table 1 The demographic and clinical characteristics of the study groups

	No symptoms $n = 198$	Mild or severe symptoms $n = 85 + 27 = 112$	p	Univariate model OR (95% CI)	Multivariate model[a] OR (95% CI)
Age (years)	29.0 (SD 4.5)	30.5 (SD 4.9)	0.015		
Age > 30	76 (38.4%)	59 (52.7%)	**0.007**	**1.79 (1.12–2.86)**	**1.75 (1.07–2.86)**
BMI (kg/m^2)	24.1 (SD 4.6)	23.8 (SD 3.9)	0.456		
BMI > 30	21 (10.6%)	8 (7.1%)	0.314	0.65 (0.28–1.52)	0.67 (0.28–1.60)
Induction of labor	37 (18.7%)	28 (25.0%)	0.190	1.45 (0.83–2.53)	1.36 (0.76–2.43)
Oxytocin for augmentation	162 (81.8%)	94 (83.8%)	0.638	1.16 (0.62–2.16)	0.90 (0.47–1.73)
Duration of 2 stage					
≤ 5 min	6 (3.0%)	5 (4.5%)	0.017	1.97 (0.58–6.73)	2.06 (0.55–7.72)
5.01–44.99 min (ref)	130 (65.7%)	55 (49.1%)		1	1
≥ 45 min	62 (31.3%)	52 (46.4%)		**1.98 (1.22–3.22)**	1.41 (0.82–2.42)
Episiotomy	107 (54.0%)	73 (65.2%)	0.056	1.59 (0.99–2.57)	1.23 (0.71–2.23)
Epidural analgesia	138 (69.7%)	77 (68.8%)	0.862	0.96 (0.58–1.58)	0.81 (0.48–1.39)
Spinal analgesia	17 (8.6%)	8 (7.1%)	0.654	0.82 (0.34–1.96)	0.77 (0.30–1.98)
Birthweight (grams)	3742 (SD 493)	3704 (SD 480)	0.515		
weight > 4 kg	50 (25.3%)	31 (27.7%)	0.640	1.13 (0.67–1.91)	1.23 (0.71–2.14)
Head circumference mean cm	35.6 (SD 1.4)	35.3 (SD 1.5)	0.117		
Occipitoposterior position	21 (10.6%)	21 (18.8%)	**0.044**	**1.95 (1.01–3.75)**	1.81 (0.91–3.61)
Instrumental delivery	65 (32.8%)	57 (50.9%)	**0.002**	**2.12 (1.32–3.41)**	**2.06 (1.26–3.36)**
Classification of injury					
3a (ref)	100 (50.5%)	39 (34.8%)	**0.008**	**1.91 (1.18–3.08)**	**1.92 (1.17–3.15)**
3b/c/4	98 (49.5%)	73 (65.2%)			
Operation technique end-to-end (ref overlapping)	123 (62.1%)	58 (51.8%)	0.076	0.66 (0.41–1.05)	0.86[b] (0.50–1.50)
Operated by a resident alone (ref specialist)	42 (21.2%)	17 (15.2%)	0.194	0.67 (0.36–1.23)	0.69 (0.36–1.30)

Statistically significant p values are shown in bold

Data presented as mean (SD) or n (%)

[a]Adjusted for maternal age, BMI, parity, mode of delivery

[b]Also adjusted for classification of injury

doctor varied from 1 single assisted operation to 31 performed operations. The operative technique was overlapping for 134 (41.2%) and end-to end for 191 (58.8%) patients, according to the operating surgeon's preference. The end-to-end technique was used mostly (92.6%) for stage 3a or 3b ruptures, and all of the stage 4 ruptures were treated using the overlapping technique.

Anal incontinence symptoms

According to the three-choice assessment, 198 (63.9%) women reported no symptoms at 6 months after OASIS repair and did not wish for an appointment with a doctor. Mild symptoms without need for a visit were reported by 85 (27.4%) women. Severe symptoms were reported by 27 (8.7%) women.

As shown in Table 1 (univariate model), advanced maternal age, long duration of the second stage of the delivery, occipitoposterior presentation, instrumental delivery, severe type of injury, and hospital stay of over 4 days were all associated with poorer outcomes at 6 months. However, in the multivariate model, only instrumental vaginal delivery, severe injury, and advanced maternal age persisted in being associated factors. Neither the operative technique nor the experience or specialization of the operating physician (resident gynecologist, specialist in gynecology or colorectal surgeon) was associated with poor outcome. The milder 3a tears were more often managed by a resident (34.5% of all 3a tears), whereas all the grade 4 tears were operated on by a senior gynecologist. Only 26 (8.1%) of the anal sphincter ruptures were operated on by a colorectal surgeon. Three out of 27 (11%) patients with persistent severe symptoms had a second sphincter repair operation. One of the patients regained anal continence after the operation, while two remained incontinent. Most of the remaining 24 patients received extended pelvic floor physiotherapy and were satisfied with the results.

Wexner incontinence score

The Wexner incontinence score was returned by 227 patients (70.1% of the total study population). The Wexner incontinence score was 0–4 for 193 (59.9%) patients, 5–6 for 17 (5.3%), and ≥ 7 for 17 (5.3%) patients. In the asymptomatic group, the Wexner score was 0–4 for 120 patients (99.2%), while 22 patients (81.5%) of those with severe symptoms had a Wexner score of 5 or higher. However, there was some discrepancy between the Wexner score and the patients' perception of the severity of their symptoms; in the asymptomatic group, the Wexner incontinence score ranged from 0 to 9, and in the mildly or severely dissatisfied groups from 2 to 14. Nevertheless, the three-choice assessment and the Wexner incontinence score showed significant correlation (Spearman's rho 0.82) (Fig. 2).

Discussion

The overall short-term subjective results of OASIS primary repair were encouraging. However, 9% of the patients had severe symptoms at 6 months. This is similar to results of other studies, although other studies do not take into account subjective patient factors [15].

We found the grading of the injury to be an important prognostic factor. This finding supports that of Roos et al., where patients with grade 3c–4 injuries had a significantly poorer outcome than those with grade 3a–b injuries based on quality of life and the results of anal manometry. Women with major tears were also significantly more likely to have an internal and external anal sphincter defect detectable by endosonography [16].

Instrumental vaginal delivery was independently associated with AI at 6 months after primary sphincter repair. Johannessen et al. [17] discovered the occipitoposterior presentation to be the only prognostic factor for AI in primiparas. However, in our study, the association between the occipitoposterior position and AI disappeared after adjusting for maternal age, BMI, parity, and mode of delivery in multivariate logistic regression analysis. Neither was the duration of the second stage of delivery a prognostic factor for poor outcome. A mediolateral episiotomy has been considered as a protective factor from OASIS [18], but, in our study, the episiotomy did not seem to have an effect on AI symptoms after primary repair.

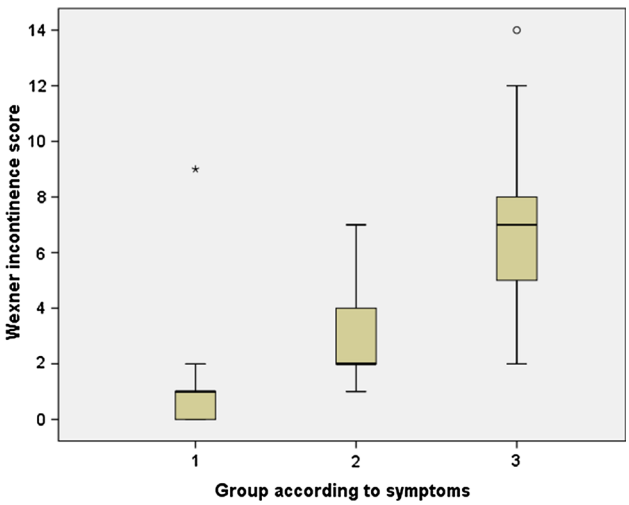

Fig. 2 The correlation between the Wexner incontinence score and the three-choice assessment

There was no statistical difference between the two repair techniques at 6 months. This is in contrast with some studies that found the overlapping technique was superior according to the symptoms at 12 months [19]. However, the milder ruptures were more often repaired using the end-to-end technique and the more severe ruptures with overlapping. The specialization or seniority of the surgeon did not seem to affect the results, although there was probably bias due to differences in assessment of the severity of the injury. The crucial step in OASIS treatment may be the recognition, and the operative technique seems to be less important.

The Wexner incontinence score is a simple and well established grading system [20]. The Wexner score of 9 has been considered a threshold for severe AI with significant impairment of the quality of life associate with scores higher than this [21]. Our study showed that patients with a Wexner score of 5 or higher reported severe symptoms. Therefore, a Wexner score of 4 might be a suitable threshold for further examinations to detect poor responders after primary repair in OASIS patients (young, previously healthy women). Additionally, in our study population, a simple three-choice assessment was very informative, as the correlation between the three-choice assessment and the Wexner score was good. The three-choice assessment takes the patient's desire for further action into consideration and is informative especially when combined with the Wexner score. A visual analog scale (VAS) has also been used together with St Mark´s score to detect women who are troubled by AI [22]. Devesa et al. did not find sufficient agreement between the Wexner score and VAS and did not recommend the replacement of the validated AI scores [23]. In our opinion, the three-choice assessment might be a useful addition to current assessment methods.

The incidence of OASIS remained low during the study period, in contrast to some reports from Australia [24] and the UK [25]. The low incidence in Finland might be due to the practice of manually supporting the perineum when the baby's head is crowning through the vaginal introitus [11]. In Israel, the incidence of OASIS has remained low, in spite of increased detection rate, due to the incorporation of manual perineal protection, the avoidance of midline episiotomy and the fact that the use of forceps is almost extinct [26]. In our study population, the recurrence rate of OASIS was 5%, which was similar to previous findings [27].

The long-term outcome of anal sphincter repair has to be further investigated. Farrell et al. found in their randomized trial comparing operative techniques that 39% (end-to-end) to 61% (overlapping) of primiparas suffered from flatus incontinence at 6 months, and at 3 years, flatus incontinence still bothered 39 and 43% of women, respectively [28]. In a Dutch cohort study, the women with OASIS had more than double the risk of long-term troublesome AI compared with the control group after a 4-year follow-up [29].

There are still women who need alternative treatment methods after OASIS primary repair. Secondary sphincteroplasty may not have the desired results, as the women often have denervated sphincters [30]. Sacral nerve modulation, as well as transcutaneous posterior tibial neuromodulation, has successfully been used for fecal incontinence, although follow-up times have been short [31]. Tissue engineering and stem cell therapy are future options and open new possibilities for the restoration of both the anatomy and function of the anal sphincter muscle [32–34].

This study reflects the usual practice in a large teaching hospital where over 60 doctors operated on OASIS patients during the study period. That outcome seems unrelated to specialty or experience deems not to support concentration of repair to the hands of a few.

Among the strengths of the study are the low drop-out volume, and the fact that a single center study enables thorough checking of every patient file to guarantee accurate classification of data. Our results reflect the patient's own perception of the problem and the desire for further action, which might help in targeting health care resources. One limitation of the study is that not all patients returned the questionnaire and the Wexner score was missing in 27% of the cases. Other limitations are the retrospective nature of the study, as well as the lack of objective measurements (ultrasound, physical examination) of all the patients. Additionally, the three-choice assessment was not validated.

Conclusions

Six months after delivery severe symptoms after OASIS repair were present in 9% of women. In addition to the Wexner score, a simple three-choice assessment of anal incontinence symptoms might be useful for evaluating the results of anal sphincter primary repair in OASIS patients and to survey the patient's desire for further procedures.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the Tampere University Hospital Science center according to the Finnish legislation and Helsinki declaration.

Informed consent For this type of study, formal consent is not required.

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ABSTRACT

Anal incontinence is a devastating condition that significantly reduces the quality of life. Our aim was to evaluate the effect of human adipose stem cell (hASC) injections in a rat model for anal sphincter injury, which is the main cause of anal incontinence in humans. Furthermore, we tested if the efficacy of hASCs could be improved by combining them with polyacrylamide hydrogel carrier, Bulkamid. Human ASCs derived from a female donor were culture expanded in DMEM/F12 supplemented with human platelet lysate. Female virgin Sprague-Dawley rats were randomized into four groups (n = 14–15/group): hASCs in saline or Bulkamid (3×10^5/60 µl) and saline or Bulkamid without cells. Anorectal manometry (ARM) was performed before anal sphincter injury, at two (n = 58) and at four weeks after (n = 33). Additionally, the anal sphincter tissue was examined by micro-computed tomography (µCT) and the histological parameters were compared between the groups. The median resting and peak pressure during spontaneous contraction measured by ARM were significantly higher in hASC treatment groups compared with the control groups without hASCs. There was no statistical difference in functional results between the hASC-carrier groups (saline vs. Bulkamid). No difference was detected in the sphincter muscle continuation between the groups in the histology and µCT analysis. More inflammation was discovered in the group receiving saline with hASC. The hASC injection therapy with both saline and Bulkamid is a promising nonsurgical treatment for acute anal sphincter injury. Traditional histology combined with the 3D µCT image data lends greater confidence in assessing muscle healing and continuity.
STEM CELLS TRANSLATIONAL MEDICINE *2018;7:295–304*

SIGNIFICANCE STATEMENT

The increasing awareness of good quality of life sets demands for better and less invasive treatment methods of the pelvic floor disorders, for example, anal incontinence. Human adipose stem cells (hASCs) are readily available, easily obtained, have low immunogenicity and high multilineage differentiation and are, therefore, an ideal cell source. In this study, an animal model was used to develop a mini-invasive injection treatment method using hASCs. The functional measurements showed significant improvement in hASC-treatment groups compared with the controls. A biocompatible carrier polyacrylamide hydrogel Bulkamid was found to be a suitable carrier for the stem cells, and a novel method of micro-computed tomography was found useful for targeting the histological slides.

INTRODUCTION

Anal incontinence (AI) is a devastating condition that significantly reduces the quality of life. Especially in women, the fecal incontinence symptoms cause depression, embarrassment and lifestyle changes that have a negative effect their everyday life [1]. The primary management of AI is conservative treatment including dietary, medical, and psychological interventions, as well as physiotherapy. A common operation for persistent AI has been secondary sphincteroplasty which may not have the desired long-term results, especially with denervated sphincters [2]. Sacral nerve modulation and transcutaneous posterior tibial neuromodulation have increasingly been used for fecal incontinence. However, the cure rates vary depending on the outcome measure in use with long-term results reaching up to 54% for sacral neuromodulation [3, 4]. Surgical methods often carry a risk of complications and are both demanding and expensive.

AI is defined as an involuntary loss of flatus, liquid, or solid stool that is a social or hygienic

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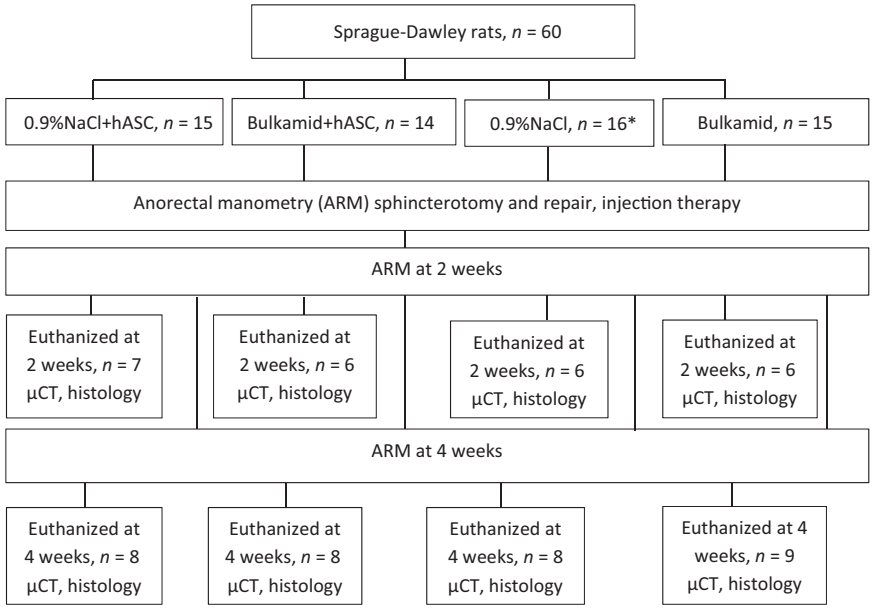

Figure 1. Flow chart of the study protocol. *2 rats died presumably due to anesthesia complication. Abbreviations: hASCs, human adipose stem cells; μCT, micro-computed tomography; NaCl, sodium chloride.

problem [5]. There are different estimates of the incidence of AI varying from 3% to over 40% in the adult population, depending on the definition and study population [6–8]. In women, obstetric injury is one of the main causes of AI, although its etiology is often multifactorial [9].

There are several studies concerning stem cells in the treatment of anal sphincter defects. Rat models with muscle-derived stem cells or myoblasts as the stem cell origin are the most common study designs [10–14], although bigger animals (rabbits, dogs) have been used as well [15, 16]. Mesenchymal stem cells (MSC) derived from bone marrow have been used in a few studies and the results are promising [17–19]. Additionally, a small clinical trial with human adipose stem cells (hASCs) has recently been published [20].

Human ASCs are easily obtained, and their features include low immunogenicity and high multilineage differentiation capability [21]. The ASCs have shown to differentiate into muscle cells [22, 23]. Human ASCs have also demonstrated paracrine function—immunomodulation, cytokine secretion, cytoprotection, and neovascularization [21, 24, 25]. Therefore, ASCs are an attractive source of cell material for regenerative medicine and tissue engineering to rehabilitate impaired organ function. However, there is a need for optimal carrier material to provide a favorable environment for ASCs' paracrine actions. Bulkamid is a nondegradable viscoelastic water-based polymer and it is mainly used for injection therapy of female urinary incontinence [26, 27]. It has also been used for AI [28]. Other bulking agents, such as bovine collagen, have been tested but the results are transient [29, 30].

The purpose of our study was to develop a novel, minimally invasive, and effective treatment method utilizing hASCs combined with either saline (0.9% NaCl) or polyacrylamide hydrogel (Bulkamid) to cure AI due to acute anal sphincter trauma. To facilitate clinical translation, cells of human origin were used and hASCs were isolated and expanded in clinically viable conditions using human platelet lysate as a culture medium supplement instead of fetal bovine serum. This is, to our knowledge, the first study to use Bulkamid as a stem cell carrier, to study in vivo the hASCs in an animal functional model and to use micro-computed tomography (μCT) as a tool to view the treated anal sphincter area.

Materials and Methods

The animal study protocol was approved by the Regional State Administrative Agency (AVI/Ella no ESAVI/2828/04.10.07/2015). Adipose tissue sample was obtained under the approval of the Ethics Committee of the Pirkanmaa Hospital District (Tampere, Finland, R15161). The hASCs were isolated from adipose tissue sample from a female donor undergoing elective plastic surgery at Tampere University Hospital (Tampere, Finland) with the patient's written consent. The final study was designed after a pilot study of 24 Sprague-Dawley rats (results not included in the analysis). In the pilot study, the rat anorectal manometry (ARM) technique, anal sphincter cutting and repairing, the injection technique, the suitable amount of the gel, and the amount of stem cells per injection were tested. The cell amounts of 5×10^5/100 μl and 5×10^6/100 μl were compared, and the lower cell count was chosen due to better cell viability before injection.

Treatment Protocol

Sixty (60) Sprague-Dawley female virgin rats (Janvier Laboratoires), weight 220–300 g, age 14 weeks were randomly selected, then anesthetized with intraperitoneal injections of medetomidine 0.25 mg/kg and ketamine 32.5 mg/kg, and buprenorfin 0.05 mg/kg s.c. and carprofen 5 mg/kg s.c. were used for postoperative pain. After the anal manometry and the sphincter operation, the anesthesia was reversed using atipamezole 1 mg/kg.

The ARM was performed using Polygraf ID manometry system with ERCP manometry triple lumen catheters (Medtronic, Polygram NET, computer unit Windows XP, Minneapolis, MN, USA). The measurements were conducted preoperatively and at 2 and 4 weeks after the operation. (Fig. 1, Supporting Information Fig. S1).

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The duration of ARM procedure was approximately 30 minutes, during which resting anal sphincter pressures and peak pressures during spontaneous contraction were measured (resting pressure 3–7 times [mean 5.64] and contraction pressure 7–12 times [mean 10.4]). Mean values of these measurements were used in further analysis.

The sphincter was cut from all animals to mimic an acute fourth grade anal sphincter tear (injury of the external and internal sphincter muscle and anal mucosa) and sewed back with 6-0 poliglecaprone (Ethicon, Johnson & Johnson, Monocryl, Somerville, NJ, USA) continuous stiches utilizing magnifying loupes. First, the anal mucosa and internal sphincter were repaired, and then, the injections were delivered after which the perineal skin was closed.

The rats were divided into four groups for the injections: injection of 3×10^5 hASCs in saline (0.9% NaCl) solution ($n = 15$), hASCs in 2.5% polyacrylamide hydrogel (Bulkamid, Contura International A/S, Denmark) ($n = 15$). The control groups consisted of rats with only saline ($n = 16$, two died within the first postoperative day presumably because of anesthetic reaction and were excluded from the analysis) and polyacrylamide hydrogel injections ($n = 14$). There were two injections with a 25-gauge needle on both ends of the external sphincter (degrees 30° and 330° on superimposed clock face, Supporting Information Fig. S3); the total injection volume of the four injections being 60 μl per animal for all Bulkamid and 0.9% NaCl-groups.

The rats were again anesthetized for the anal manometry control with medetomidine and ketamine combination as described above. After the last anal manometry examination, the rats were euthanized using carbon monoxide. The anal manometry results were analyzed by a blinded researcher (HT) to exclude observer bias.

ASC Isolation, Cell Culture, Cell Viability, and Phenotype

The hASCs were isolated as previously described [31]. The hASCs were expanded in DMEM/F12 (1:1) (Thermo Fisher Scientific Inc., Carlsbad, CA, USA, https://www.thermofisher.com) supplemented with 5% Pooled Human Platelet Lysate (Stemulate; Cook General Bio-Technology, Indianapolis, IN, USA, http://cookregenterec.com), 1% antibiotics (100 U/ml penicillin and 0,1 mg/ml streptomycin; Thermo Fisher Scientific Inc.), and 1% L-glutamine (Thermo Fisher Scientific Inc, GlutaMAX-100, Indianapolis, IN, USA). The medium was changed twice a week, and the cells were divided upon reaching confluency. The cells were detached using TrypLE Select (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). From 4 to 5 days prior to cell injections, the ASCs were labeled with 200 μg/ml magnetizable nanoparticles (PMP-50; Kisker Biotech GmbH & Co., Steinfurt, Germany, https://kisker-biotech.com) for 48 hours for cell detection. For cell injections, 5×10^5 hASCs (passage between 4 to 7) were blended with 100 μl of 0.9% NaCl (Baxter Healthcare SA, Zurich, Switzerland, http://Baxter.com) or Bulkamid hydrogel (Contura International A/S, Soeborg, Denmark, https://bulkamid.com).

For the cell viability, 5×10^5 hACSs were blended with 100 μl Bulkamid, incubated at room temperature for 3 hours (the maximum delay between the cell preparation and the injections), and the cell viability was assessed with LIVE/DEAD Viability/Cytotoxicity kit for mammalian cells (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). The hASCs in Bulkamid were stained with 1 μM Calcein AM and 0.8 μM Ethidium homodimer-1 (EthD-1) for 45 minutes. Fluorescence pictures were taken with Fluorescence Microscope (Olympus. IX51S18F-2 and camera DP71, Japan).

The phenotype of the hASCs was assessed with fluorescence-activated cell sorter (BD Biosciences, Franklin Lakes, NJ, USA, https://www.bdbiosciences.com. BD FACSAria Fusion Cell Sorter) at passage 7. Monoclonal antibodies against CD14-phycoerythrincyanine (PE-Cy7), CD19-PE-Cy7, CD45RO-allophycocyanin (APC), CD54-Fluorescein isothiocyanate (FITC), CD73-phycoerythrin (PE), CD90-APC (BD Biosciences, Franklin Lakes, NJ, USA), CD105-PE (R&D Systems Inc., Minneapolis, MN, USA, http://www.rndsystems.com), CD34-APC and HLA-DR-PE (Immunotools GmbH, Friesoythe, Germany, http://www.immunotools.de) were used. According to the International Society for Cellular Therapy standard criteria, cells positive for CD73, CD90 and CD105 but negative for CD14, CD34, CD45, and HLA-DR are considered as MSCs [32, 33].

Table 1. Technical details about the micro-computed tomography imaging

Voltage	60 kV
Current	166 μA
Source distance	58 mm
Detector distance	170 mm
Exposure time	4 seconds
Imaging time	2.5 hours
Pixel size	17.0354 μm
Image volume dimensions	1000 × 1000 × 1000 pixels

μCT Imaging

The analysis of the anal sphincter was performed after sacrificing the animals. The excised samples were imaged in a μCT instrument (Xradia MicroXCT-400, Carl Zeiss, Pleasanton, CA, USA). To increase the contrast between the soft tissues, the samples were put through a staining regimen. The tissue samples were stored in 4% paraformaldehyde (PFA)-solution (Sigma). The PFA was then changed to 70% ethanol solution, then to 95% ethanol, ≥99.5% ethanol, and 10 mg/ml iodine in ethanol. The samples were transferred into sample holders and were kept at room temperature for a minimum of 24 hours prior to imaging in order to avoid imaging artifacts resulting from thermal expansion of the tissue samples. The imaging parameters for all 58 samples were constant (Table 1). After imaging, the samples were transferred into a 70% ethanol solution for storage and following histological analyses. Tomographic three-dimensional (3D) image reconstruction was performed with proprietary software installed in the instrument (Carl Zeiss, Xradia XMReconstructor, Pleasanton, CA, USA). The reconstructed image volume was imported into AVIZO image processing software (FEI Company, Hillsboro, OR, USA) for image processing and inspection. The anal sphincter complex was visualized through 3D volume rendering. It was used in combination with tomographic data exploration as freely selectable two-dimensional (2D) views of the 3D dataset to confirm the histological assessment of muscle continuity. Additionally, it was used to visualize in 3D the entire sample instead of selected 2D sections as in histology and to monitor the presence of any detectable PMP-50 labeled cells.

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Histology

The formalin fixed, paraffin embedded tissues were sectioned (4 μm) and stained with Hematoxylin & Eosin (HE) for morphological interpretation. Furthermore, picrosirius red staining was used to demonstrate collagen and Perls Prussian blue to demonstrate the presence of iron from tissue sections. Immunohistochemistry using anti-human Vimentin 1:200 (Clone:BS13, BSH-7100, Nordic Biosite, Täby, Sweden) and STEM121 1:500 (Clone: Stem121, Y40410, Takara Bio Inc., Shiga, Japan) antibodies was performed to detect hASCs from rat tissue sections. Smooth muscle actin antibody 1:200 (Clone BS66, BSH-7459, Nordic Biosite, Täby, Sweden) and Anti-Desmin mouse monoclonal antibody (Clone BS21, Nordic Biosite, Täby, Sweden) were used to detect rat muscle tissue. Finally, CD68 antibody 1:200 (Clone: ED1, ab31630, ABCAM, Cambridge, United Kingdom) was used to detect rat macrophages and verify the origin (PMP-50 containing hASCs or naturally iron containing rat macrophages) of positive Perls Prussian blue staining. All antibodies were produced in mouse and primary antibodies were detected using anti-mouse horseradish peroxidase polymer. Rat and human paraffin control multi-tissue sections were used as a negative and positive tissue controls for immunohistochemical analyses.

Olympus BX-60 microscope (BSH 747) and an integrated color digital camera (Scion) were used for the evaluation of the slides. The slides were also imaged and digitalized with a 3D Histec Pannoramic MIDI instrument. The scoring of the inflammation was based on the inflammatory cell infiltration into the lesion site, edema, hemorrhage and necrosis of tissue [34]. The number of inflammatory cells was evaluated manually from the low power field (×200) image frame using a "hot spot" selection. The inflammation was scored with following grades: 0 = no histological features of inflammation; 1 = diffuse inflammatory cell infiltration, <100 cells; 2 = diffuse inflammatory cell infiltration, 100–500 cells, mild edema and hemorrhage; 3 = inflammatory cell infiltration, >500 cells, edema and hemorrhage; 4 = inflammatory cell infiltration, >1,000 cells, edema, hemorrhage and necrosis. Fibrosis was evaluated separately with following grades: 0 = no fibrosis; 1 = mild fibrosis/collagen formation; 2 = strong fibrosis/collagen formation. The infiltration of the cells into the hydrogel was evaluated with samples containing Bulkamid.

Statistical Analysis

The statistical analysis was performed by using the IBM SPSS version 22 (IBM, Chicago, IL, USA). The four treatment groups were compared at baseline, at 2, and at 4 weeks. Statistical significance was tested by using chi-square or Fisher's exact test with categorical and one-way analysis of variance (ANOVA) with continuous variables. The groups and the time points within the groups were compared using ANOVA for repeated measures.

Results

In Vitro Results

Live/dead staining demonstrated that after 3 hours of incubating the hASCs in Bulkamid, the hASCs were mostly alive. However, there were also some dead cells in the mixture (Fig. 2). The hASC phenotype was assessed at passage 7 using flow cytometry. The hASCs expressed surface markers CD73 (99.9%), CD90 (99.9%), and CD105 (99.6%). Expression of CD14 (1.4%), CD45RO (0.6%), and HLA-DR (0.3%) was very low, and expression of CD19 (6.0%), CD34 (8.1%), and CD54 (8.5%) was low. This confirms the mesenchymal origin of the hASCs.

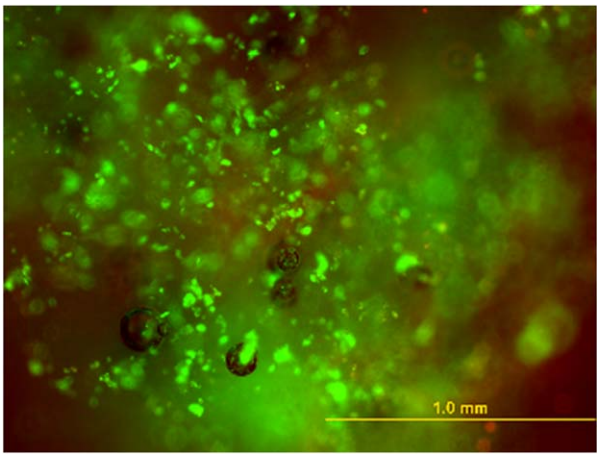

Figure 2. Live/dead staining of the human adipose stem cells in Bulkamid; dead cells (red), live cells (green). Scale bar 1.0 mm.

In Vivo Results

The baseline rat characteristics (weight, ARM measurements) of each group are presented in Table 2. The rats in the 0.9% NaCl and Bulkamid control groups were slightly, though statistically significantly, heavier than the hASC treatment groups' rats. Otherwise, there was no difference in baseline characteristics between the groups.

Anorectal Manometry Results

First, the four groups were compared based on the ARM results before injury and at 2 and 4 weeks. The measured variables were the median resting pressure and the peak pressure during spontaneous contraction of the anal sphincter complex. The median resting and the peak contraction pressures were higher in the hASC treatment groups at 2 and at 4 weeks (Table 2). Further analysis showed that the trend of the contraction pressure was significantly higher in the both hASC-groups compared with the saline and Bulkamid control groups (Fig. 3). The difference between the groups remained statistically significant when adjusted for baseline measurement.

Histology

In the histological analysis, no hASCs were recognized in the preparations neither at 2 nor 4 weeks according the Vimentin, STEM121, or Perls Prussian blue staining. This was confirmed with CD68 staining of rat macrophages. Rat endogenous macrophages containing iron stained positively with Perls Prussian blue and rat specific CD68 immunoperoxidase reaction located into the same cells (Fig. 4). Vimentin and STEM121 were positive in a cytoblock section prepared from the same cells that were injected into the rats (data not shown).

There was no statistical difference in sphincter muscle continuity, fibrosis, or collagen formation between the four groups. The Bulkamid-hydrogel was well integrated in the tissue with minor foreign body reaction according to the HE staining. There was more inflammation in the hASC-groups, especially in the 0.9% NaCl +hASC-group (Table 2; Fig. 4, Supporting Information Fig. S2).

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Table 2. The rat baseline characteristics, functional results from anorectal manometry and histology

	0.9%NaCl + hASC		Bulkamid+hASC		0.9%NaCl		Bulkamid		
	Mean/median	SD/Q_1–Q_3	Mean/median	SD/Q_1–Q_3	Mean/median	SD/Q_1–Q_3	Mean/median	SD/Q_1-Q_3	*p* value
Weight (g)	268.0/ 270.0	12.1/ 260.0–280.0	263.6/ 270.0	18.6/ 250.0–280.0	275.4/ 280.0	15.7/ 267.5–290.0	285.3/ 280.0	11.4/ 280.0–300.0	.002
PreopARM (*n*)	15		14		14		15		
Rest med	9.1	3.4	8.4	2.8	7.8	2.4	8.2	2.3	.640
Peak contr	97.5	25.2	88.5	29.6	81.3	22.1	85.0	23.3	.349
ARM 2 wk (*n*)	15		14		14		15		
Rest med	9.3	2.6	9.6	2.1	4.9	3.2	5.2	1.6	< .000
Peak contr	74.6	16.0	74.0	13.6	44.6	20.6	47.8	14.3	< .000
ARM 4 wk (*n*)	8		8		8		9		
Rest med	10.1	3.0	9.2	2.3	5.4	2.5	6.1	3.4	.005
Peak contr	79.5	16.4	76.2	21.3	52.4	27.2	46.6	26.7	.014
Inflammation									.003
gr 0 (%)	0.0		21.4		28.6		33.3		
gr 1 (%)	20.0		28.6		50.0		46.7		
gr 2 (%)	40.0		42.9		21.4		20.0		
gr 3 (%)	13.3		7.1		0.0		0.0		
gr 4 (%)	26.7		0.0		0.0		0.0		

Abbreviations: ARM 2 wk, anorectal manometry at 2-week time point; ARM 4 wk, anorectal manometry at 4-week time point; hASC, human adipose stem cells; NaCl, sodium chloride; Peak contr, peak pressure during spontaneous contraction; Preop ARM, preoperative anorectal manometry; Q_1–Q_3, 25 and 75 percentiles; Rest med, median resting anal pressure; Weight, preoperative weight.

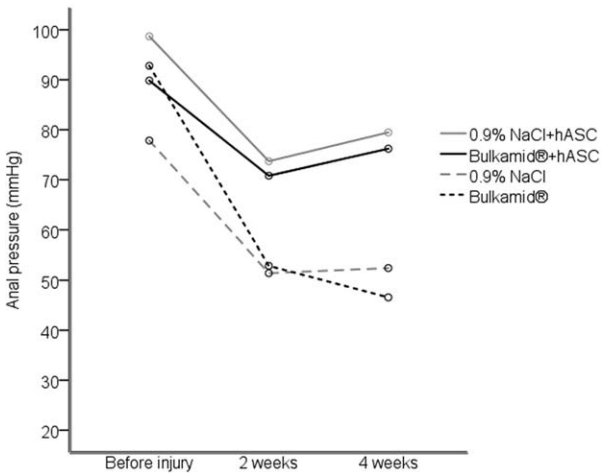

Figure 3. The trends of the four groups showed a significantly higher contraction pressures in both hASC treatment groups. Abbreviations: hASCs, human adipose stem cells; NaCl, sodium chloride.

μCT Analysis

The μCT image datasets were used to confirm independently the continuity of the sphincter muscle shown in the histology. By viewing the image data in multiple orthogonal views, greater confidence could be attested to the histological assessment. (Fig. 5, Supporting Information video). There was total agreement between histology and μCT interpretation in 76% of the samples. There was minor disagreement in 11 samples and serious disagreement in muscle continuity in 3 samples (5%). This did not affect the statistical difference between the groups. Thus, the ability to conduct nondestructive histomorphometric analysis on samples provides valuable image data that can be used to perform robust 3D analyses when necessary. In some samples with Bulkamid, small regions with high x-ray attenuation regions were observed that could indicate cells or remains of PMP-50 particles (data not shown). However, it was not possible to confirm whether these regions indicate the presence of PMP-50 particles or whether they were the result of local aggregation of iodine. As iron specific staining of the histological samples failed to detect PMP-50 in any of the samples, the presence of PMP-50 particles could not be confirmed.

Discussion

Our aim is to develop an effective, mini-invasive treatment for AI, which is a highly distressing condition and all the more lacking an efficient treatment method. Studies about the existing treatment methods are heterogeneous and long-term results are mostly missing. Tissue engineering and cell therapy have been considered to be compelling alternatives and hold a great deal of promise and excitement. The advantage of using ASCs compared with the other stem cell sources is that the tissue can easily be harvested and it is readily available in large quantities. ASCs can be easily expanded in vitro and have an extensive self-renewal capacity [35, 36].

In our in vivo-study, we found significant improvement in anal sphincter resting and contraction pressures in hASC treatment groups after acute sphincter injury compared with the control groups. Previous functional anal sphincter assessments have mostly been performed in vitro using anal sphincter muscle samples from euthanized animals to measure the contractility after electrical stimulation [10, 12, 17, 37]. Salcedo et al. measured in

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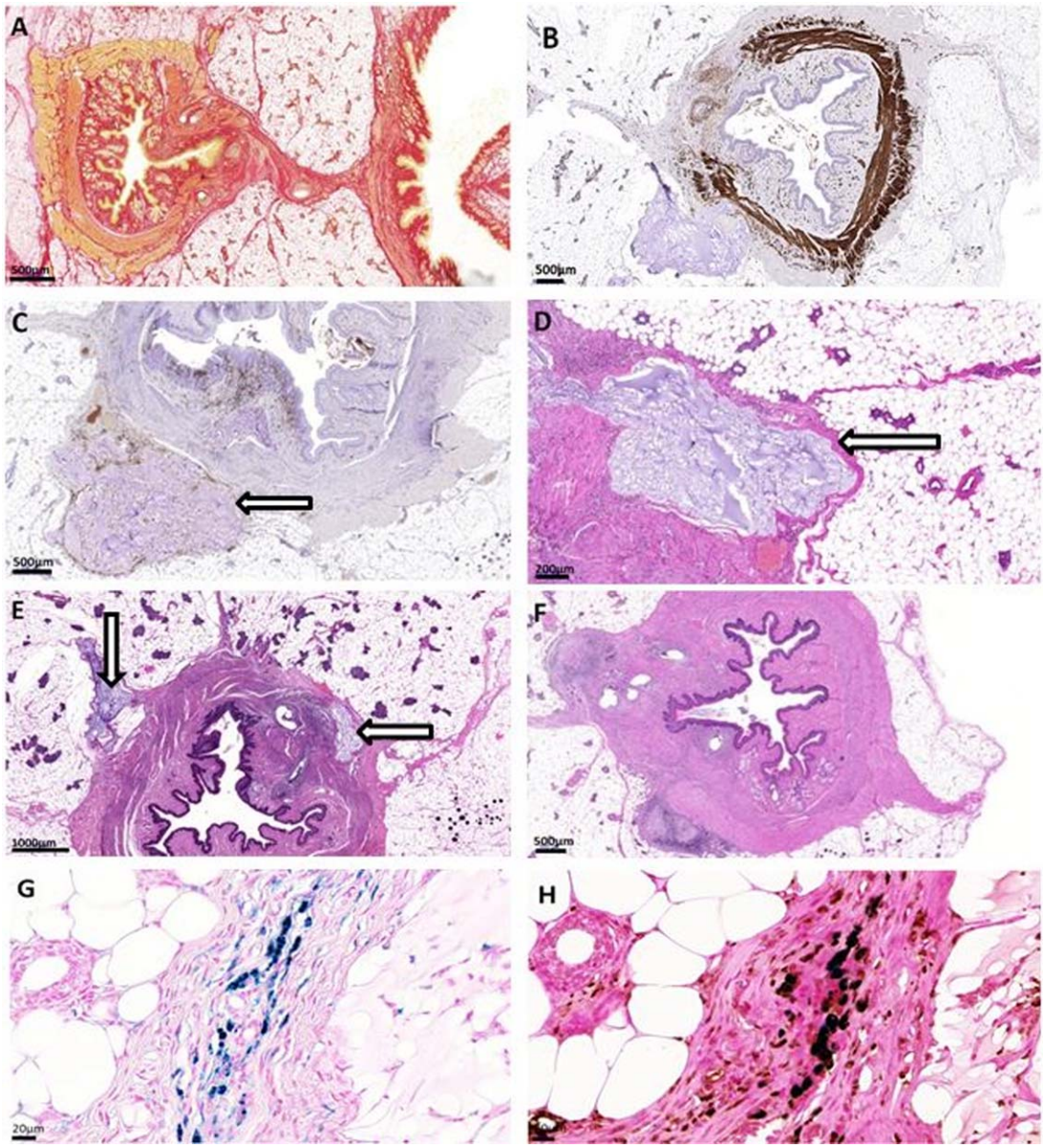

Figure 4. The histology stainings and examples of the inflammatory grading. **(A)**: Picosirius red-staining, 0.9% sodium chloride (NaCl) at 2 weeks; **(B)**: Anti-Desmin, Bulkamid at 4 weeks; **(C)**: Immunohistochemistry staining CD68, Bulkamid+hASC at 2 weeks; **(D)**: HE-staining, Bulkamid+hASC at 2 weeks, inflammation grade 1; **(E)**: HE-staining, Bulkamid+hASC at 4 weeks, inflammation grade 2; **(F)**: HE-staining, 0.9%NaCl + hASC at 2 weeks, inflammation grade 4; **(G)**: Perls Prussian blue-staining for iron particles, Bulkamid+hASC at 2 weeks; **(H)**: Combination of CD68 and Perls Prussian blue of the sample G showing that the iron particles localize at the rat endogenous macrophages. Arrow = Bulkamid+hASC-injection. Scale bar Figures (A–F) 500 µm, Figures (G–H) 20 µm.

vivo function of the anal sphincter after a sphincterotomy or pudendal nerve crush followed by either rat bone-marrow derived MSC injection or saline [18]. In their further investigations, the same research group found that both serial i.v. infusion and i.m. injections of MSCs after partial sphincter excision resulted in increased anal pressures [19]. Pathi et al., on the other hand, did not find significant advantage in the iv-administration of rat bone-marrow derived MSCs compared with the PBS controls, but the local injections of MSCs were effective according to in vitro assessment [17]. Recently Fitzwater et al. noticed that the administration of rat myogenic stem cells enhanced the contractile function of the sphincter without significant changes in histologic morphology, which addresses the paracrine processes in stem cell therapy [14]. To simplify the cell isolation and proliferation process, Mazzanti et al. used freshly isolated minimally manipulated bone-marrow derived mononuclear cells without expansion and found them to be as effective as in vitro expanded BM-MSCs in the recovery of iatrogenic anal sphincter rupture [38]. Muscle-derived stem cell injections have been used in small pilot studies in women and the results are promising [39–41]. Frudinger et al. treated 10 patients with autologous myogenic stem cells and found a significant improvement in the AI symptoms at 1- and 5-year controls. However, there was no significant change in anal manometry measurements [39, 40]. In a small pilot study, where hASCs therapy was used in combination with surgical sphincteroplasty in treatment of AI, there was no difference in AI symptoms

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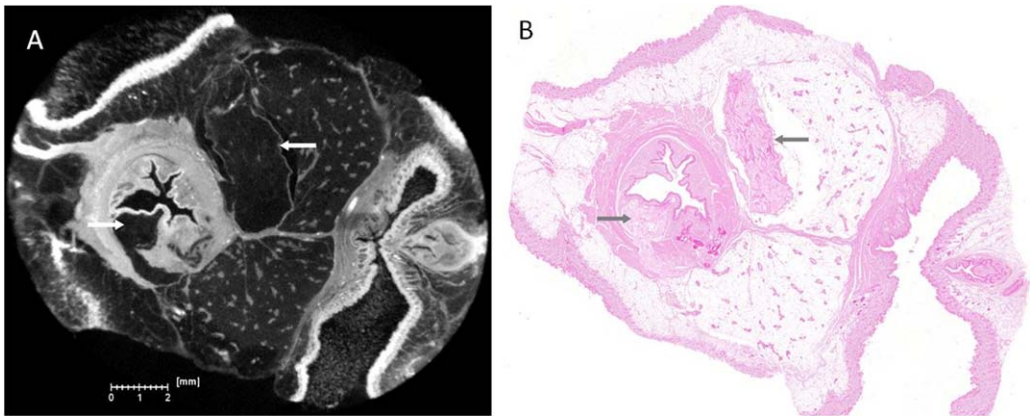

Figure 5. Comparison of the micro-computed tomography **(A)** and histology **(B)** images. Arrow = injected Bukamid hydrogel.

between the hASC and saline injection group. However, the number of patients was limited and heterogeneous and the follow-up time short. Despite these limitations, they discovered an increase in total muscle area in an endorectal sonography assessment in the hASC treatment group compared with the saline control group [20].

In our study, the anal pressures were comparable with the other animal studies although there are slight variations—possibly due to different techniques (balloon vs. double-triple-lumen-catheter, electrical stimulation vs. spontaneous contraction) and different size of the anal canal in different species [13, 16, 19]. Salcedo et al. stated that rat anal pressures tend to return to baseline after sphincterotomy at 4 weeks even without intervention, but this does not occur after pudendal nerve transection [42]. In our results, both resting and contraction anal pressures were significantly lower in both control groups at 2 and 4 weeks compared with the hASC treatment groups. There was also a rising pressure trend within the stem cell groups that might have resulted in even higher pressures and better recovery in longer follow-up.

We used ASCs from a single donor mainly because of the safety regulations. It is known that there is some heterogeneity between different donors; for example, age, sex, body mass index, and site of harvest are known to have an effect on the properties of the ASCs [43–47]. Xenotransplantation has been used before to study development, physiology, and pathophysiology of human tissues in animal models, for example, enteric nervous system [48] and rodent models have been tested in treatment of stroke [49] and myocardial infarct [50]. However, there are differences in human and animal physiology that have to be kept in mind when interpreting the results [51].

Different labeling systems of injected cells have been used in previous studies. Kang et al. found fluorescent dye PKH-26 labeled rat myogenic stem cells in fluorescent microscopy of rat anal sphincters at one week after the injection [10]. PKH-26 label in animal autologous myogenic stem cells were also used by Kajbafzadeh (rabbit) and Oh (dog): both groups found labeled cells at 2 months and 3 months control, respectively [15, 16]. Aghaeeasfhar et al. used bromodeoxyuridine labeled human umbilical cord stem cells as well as rabbit bone marrow stem cells (BMSCs) in a rabbit model, the labeled cells were found in immunohistochemistry at 2 weeks control [52]. On the other hand, Cruz et al. demonstrated that the green fluorescent protein (GFP) labeled rat BMSCs disappear in 10 days after i.v.-administration [53]. Salcedo et al. found no visible GFP-labels in the anal sphincter after administration of i.m. or i.v. MSCs derived from rat bone marrow [18]. We attempted to find the PMP-50 labeled hASCs in the tissue samples using μCT, histology and immunohistochemistry. Although some potential signs of PMP-50 were detected in μCT, we were not able to confirm the PMP-50 labeled cells either in the μCT scan or in the histology analysis. Histological sections stained positive with Perls Prussian blue but rat macrophage specific CD68 antibody stained the same cells indicating that the signal comes from hASCs ingested by rat macrophages or just iron that macrophages contain naturally. In our pilot study, there were tracks of the injected cells in hydrogel with the higher cell amount (5×10^6 cells/100 μl) and with bigger injection volume 100 μl (Supporting Information Fig. S2). In the actual study, due to better cell viability and injection experience, we decided to use lower cell count and lower volume and were not able to verify the presence of the stem cells. The evanescence of the cells may be due to the rat immune defense destroying the human cells. This is supported by the lack of human specific STEM121 and Vimentin staining of the tissue sections.

There are also the safety considerations in cell therapy. Jacobs et al. found no evidence of myogenic stem cell migration to the liver or lung. However, they detected local ectopic foci of growth in two treated rat anal sphincters after 30 days; the small tumors were benign tumors with no nuclear abnormalities [54]. In our study, normal histology was confirmed both by 3D μCT scanning of the whole sphincter sample area, as well as by selected histological sections. The μCT is a valuable approach to visualize the entire treated area and the injection sites in 3D to target the histological slides. The ability to increase imaging resolution without proportionally decreasing sample size opens possibilities for performing analyses without destructive sample preparation. The μCT protocol applied in this study showcased this methodology by complementing the traditional histological analyses with increased amount of available data, which resulted in greater confidence in the analysis. Additionally, in combination with appropriate concentrations of labeling agents, it may also be feasible to monitor the distribution of introduced cells.

This animal model, like any other animal model, is not directly comparable with birth injury in humans. The trauma in the rat model was caused by a direct clean-cut wound whereas in

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obstetric trauma the sphincter is torn, and the tissue has suffered from prolonged hypoxia and denervation. In an obstetric trauma, there is also presence of tissue edema and blood, which makes suturing the rupture more challenging in real life. In our animal model, the rupture was easy to identify with no delay, and the operative technique was consistent with only one operator. The small scale of the rat anatomy made it difficult to distinguish the inner and outer sphincter from each other and that is why the injections were aimed at the external sphincter rather than submucosa or between the sphincters. In humans, the intersphincteric injection might be better. However, our model applies to acute injury and is perhaps not applicable for chronic conditions with fibrous anal sphincters often seen in AI patients. Fibrosis is a histological finding often seen in scar tissue and in our model the follow up time was too short to analyze the possible impact on scar tissue formation. There is evidence that cell therapy might be able to prevent vocal fold scaring in laryngeal microsurgery and fibrous keloid formation after skin incision in acute trauma model [55, 56].

The possible mechanisms of AI stem cell therapy are direct stem cell integration and muscle regeneration, the bulking effect of the cells and the carrier, the trophic effect caused by the stem cell growth factor secretion or immune modulation, and the decreased inflammation that improves tissue healing [33, 57]. The bulking effect is probably not the mechanism according to our results, because there was no advantage in the bulking agent Bulkamid compared with the 0.9% NaCl-controls, and also because there were good results with hASCs in 0.9% NaCl as well. For human AI, the bulking effect in the injection therapy is perhaps not even desirable [58]. According to our results, lack of differences in muscle continuity between the groups refers to paracrine effect rather than to direct stem cell differentiation into muscle cells. The mechanism may be through cell fusion, endogenous activation of satellite cells or some inflammatory process [59]. From the inflammatory perspective, Bulkamid seems to have an advantage over 0.9% NaCl as a carrier, although it is not clear what stage of inflammation is desirable for the healing process. Bisson et al. showed that certain amount of inflammation and acute injury is required in cell therapy: injection of myoblasts on the opposite site of cryoinjured anal sphincter did not restore the anal sphincter function, whereas the injection into the lesion itself did [60]. On the other hand, Salcedo et al. showed that systemic injection also had a positive effect; however, this was not seen in the study of Pathi et al. [17, 19]. One advantage of hydrogel over 0.9% NaCl is that the cell sedimentation and aggregation are mostly avoided with Bulkamid. Bovine collagen gel (Contigen) has also been used in clinical trials experimenting stem cell injection therapy for female urinary incontinence [61, 62].

It has been suggested that stem cell transplantation may facilitate endogenous repair even in patients with advanced age and comorbidities who have compromised repair function [13]. Therefore, stem cell therapy would be especially useful in patients with compromised anal sphincter function. Bohl et al. have successfully implanted engineered biosphincters in rabbits with iatrogenic AI to restore anal sphincter function [63]. Sphincter transplantation is however a major surgical operation. Stem cell injection therapy can be performed under local anesthesia in an outpatient setting with no major complications. According to our preliminary results, the ASC treatment is effective in treatment of an acute anal sphincter injury. In the future, animal models for chronic injury are needed to develop an effective and clinically relevant treatment method.

Conclusion

ASCs combined with either saline or synthetic nondegradable hydrogel is a novel and attractive, mini-invasive treatment method for acute anal sphincter rupture and AI. Due to low inflammatory response and good tissue integration, Bulkamid hydrogel appears to be a suitable injection agent and carrier for the ASCs. The technique of anal sphincter injection therapy is simple in an animal setting and the use of human ASCs in rat AI model is feasible. The 3D μCT is a valuable addition to the traditional histology in analyzing soft tissue samples. Further studies with suitable chronic injury animal models with longer follow-up periods are needed to confirm the functional restoration and to develop a truly effective treatment method for AI patients.

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Author Contributions

K.K.: conception and design, administrative support, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; M.J. and N.N.G.: conception and design, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; H.T.: conception and design, data analysis and interpretation, final approval of manuscript; H. H.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript; K.N. and J.H.: conception and design, administrative support, data analysis and interpretation, manuscript writing, final approval of manuscript; S.M.: conception and design, financial support, administrative support, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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Autologous Adipose Stem Cells in Treatment of Female Stress Urinary Incontinence: Results of a Pilot Study

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Key Words. Stress urinary incontinence • Adipose stem cells • Female incontinence • Tissue engineering • Injection therapy

ABSTRACT

The purpose of our study was to find out whether transurethral injections of autologous adipose stem cells (ASCs) are an effective and a safe treatment for female stress urinary incontinence (SUI). We treated five SUI patients with ASCs combined with bovine collagen gel and saline. Prior to the treatment, the ASCs were isolated from subcutaneous fat and expanded for 3 weeks in a good manufacturing practice-level laboratory. The mixture of ASCs and collagen was injected transurethrally via cystoscope. Additionally, viability, multipotency, and surface marker profile of ASCs were analyzed in vitro. We followed up with patients 3, 6, and 12 months after the injections. The primary endpoint was a cough test to measure objectively the effect of the treatment. Validated questionnaires were used to determine the subjective cure rate. After 6 months, 1 of 5 patients displayed a negative cough test with full bladder filled with 500 ml of saline. At 1 year, the cough test was negative with three patients; two of them were satisfied with the treatment and did not wish further treatment for SUI. Validated questionnaires showed some subjective improvement in all five patients. This is the first study describing the use of autologous ASCs in combination with collagen gel for female SUI treatments. Thus far, the treatment with autologous ASCs has proven safe and well tolerated. However, the feasibility and efficacy of the treatment were not optimal; therefore, additional research is needed to develop SUI injection therapies. STEM CELLS TRANSLATIONAL MEDICINE *2014;3:936–941*

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INTRODUCTION

Urinary incontinence is a common health problem affecting a large number of women. Approximately 35% of women over 18 years in Europe reported involuntary urine loss [1]. Stress urinary incontinence (SUI) is the most common type, and it is defined as involuntary loss of urine on effort or physical exertion. Urgency urinary incontinence is associated with a feeling of urgency, and mixed urinary incontinence (MUI) is a combination of the two aforementioned types [2]. SUI is further categorized as urethral hypermobility, intrinsic sphincter deficiency, or both [3]. Furthermore, the prevalence of incontinence increases with age [4].

The urethral continence control system is a vital component in stress urinary incontinence. It consists of the sphincteric unit and the vaginal support system. The urethra is a multilayered structure consisting of striated muscle, smooth muscle, connective tissue, submucosal vascular plexus, and a lining epithelium. The combined actions of these tissues serve to create a wall tension that compresses the lumen closed. Striated muscle has been shown to be responsible for one-third of the total intraurethral pressure, with another third being exerted by the urethral vascular bed, and the remaining third controlled by the smooth musculature and connective tissues [5, 6].

Midurethral slings have become first-line surgical treatments for SUI because they are minimally invasive, have excellent short-term success rates and good long-term success rates, and have a short learning curve [7]. However, there are patients who have a SUI unresponsive to operative treatment and patients who are not suitable for midurethral sling operation. Additionally, there are patients who do not want artificial synthetic material as their treatment.

Tissue engineering offers an attractive treatment method to regenerate sphincter muscle. Previously, various different cell sources, such as skeletal muscle-derived stem cells (SkMSCs), mesenchymal stem cells (derived from bone marrow [BMSCs]), and adipose stem cells (ASCs), have been studied for treating urinary incontinence. The SkMSCs and BMSCs would be a potential alternative for incontinence therapy. However, when compared with ASCs, the major limitation

Table 1. Characteristics of the patients treated with transurethral injections of adipose stem cells + Contigen

Subject	Age	BMI	Previous incontinence operation	Incontinence type	MUCP (mmHg)
Patient 1	50	27		SUI	57
Patient 2	61	25	TVT	MUI	21
Patient 3	59	41		MUI	38
Patient 4	45	27		SUI	72
Patient 5	81	31	TVT-O Bulkamid TVT	MUI	20

Abbreviations: BMI, body mass index; MUCP, maximal urethral closure pressure; MUI, mixed urinary incontinence; SUI, stress urinary incontinence; TVT, tension-free vaginal tape; TVT-O, transobturator tape.

Table 2. Objective findings of urinary incontinence in patients treated with transurethral injections of adipose stem cells + Contigen

Subject	Cough test			24-hour pad test (g)	
	Preprocedure	At 6 months	At 12 months	Preprocedure	At 12 months
Patient 1	+	+	+	0[a]	0
Patient 2	+	+	−	5	3
Patient 3	+	−	+	10	9
Patient 4	+	−	−	28	4
Patient 5	+	+	−	70	28

[a]No exercise because of a hip problem.

of SkMSCs and BMSCs is the difficulty to obtain these cells in large quantities. Furthermore, the isolation site injury for ASCs is minimal, and they are readily expanded in vitro. These features are important when considering the cell source for clinical applications. ASCs, like BMSCs, have been shown to undergo myogenic, adipogenic, osteogenic, and chondrogenic differentiation [8]. Moreover, the ASCs have been used for clinical applications in different surgical areas and are considered safe for clinical use [9].

The aim of this study was to find out whether adipose stem cells could provide a new, and possibly effective, alternative to invasive surgical treatment of SUI. To our knowledge, this was the first study of transurethral injections of autologous ASCs in combination with collagen (Contigen) in treatment of SUI or MUI.

Materials and Methods

Patients were recruited from the Tampere University Hospital outpatient clinic, and primarily they did not want the midurethral sling operation. Two of them had been operated on previously (Table 1) with unsuccessful results. The Ethics Committee of Pirkanmaa Hospital District approved the study pilot. Furthermore, all patients signed a written informed consent for the study.

After gynecological examination, diagnosis of either pure SUI or predominantly stress MUI was made according to anamnesis, a positive cough test, and urodynamic evaluations. Validated questionnaires—Urinary Inventory Stress test (UISS), Detrusor Instability Score, Incontinence Impact Questionnaire-short form (IIQ-7), and Urogenital Distress Inventory-short form (UDI-6, with scores 0–100, lower scores reflecting better quality of life) and the bother of incontinence in patients' lives according to visual analog scale (VAS) (ranging 0–10) [10–12]—were filled, and exclusion of infectious diseases (hepatitis B and C, HIV, syphilis) was conducted.

The subcutaneous fat was collected from patients' lower abdomens under local anesthesia. Approximately 0.3–0.5 dl of fat was obtained from six patients. However, because of bacterial contamination (*Propionibacterium acnes* in repeated samples), one patient did not receive treatment. In addition, 50 ml of autologous serum was obtained for the expansion of clinically used ASCs. The ASCs were then isolated and augmented as described later in this article. A mixture of ASCs and collagen (Contigen; Bard Medical, Covington, GA, http://www.bardmedical.com) was injected transurethrally via cystoscope under local anesthesia. The injections were placed directly under mucosa: 1.5 cm distal from the urethral neck at 3 and 9 o'clock, injected volume being 2.4–4 ml per patient. Two additional concomitant injections of ASCs mixed with saline solution (volume 2 ml) were performed 2 mm more distally to bring the ASCs in contact with the urethral musculature.

We followed up with patients at 3, 6, and 12 months after the injections by a gynecological examination, a vaginal ultrasonography, a cough test, a 24-hour pad test, standardized questionnaires, and urodynamic evaluations (at 6 months).

The primary outcome measure was the cough test. Other outcome measures were the 24-hour pad test, urodynamic evaluations (maximal urethral closure pressure [MUCP], and urethral stress profile), and patients' evaluations of their quality of life.

Stem Cell Isolation and Preparation for Injection

The isolation and expansion of ASC was done in a validated cleanroom (BioMediTech, University of Tampere) following European Union good manufacturing practice (GMP) quality system guidelines.

The cell isolation, expansion, karyotyping, sterility, endotoxin, and mycoplasma testing were performed as described previously [9, 13]. Briefly, the adipose tissue was minced into small pieces and digested with collagenase NB-6 (GMP grade; Serva, Heidelberg, Germany, http://www.serva.de) in a 37°C incubator for 60 minutes while mixing by pipetting up and down every 20 minutes. After centrifuging and lysing the red blood cells, the pellet was suspended in the basal medium (BM) containing 15% of autologous serum in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12; Life Technologies, Rockville, MD, http://www.lifetech.com). The isolated cells were expanded for 3–4 weeks in BM. When nearly confluent (90%), the cells were mechanically detached using a cell scraper (Nunc, Rochester, NY, http://www.nuncbrand.com; Life Technologies) and passaged. For the injection therapies, we used passages 3–4, which were the lowest possible passages to get an adequate amount of cells.

Half of the freshly isolated ASCs were blended with 2.1 ml of collagen (Contigen), and the rest of the ASCs were blended with 0.9% NaCl. The amount of cells used for injection therapy varied from 2.5×10^6 to 8.5×10^6 cells, depending on the patient. The live/dead staining was used to evaluate the viability of ASCs in Contigen prior to the injection therapy as previously described [13].

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Table 3. Quality of life/subjective findings of patients treated with transurethral injections of adipose stem cells + Contigen according to UISS, IIQ-7, UDI-6, and VAS

	UISS		IIQ-7		UDI-6		VAS	
Subject	Preprocedure (0–100)	At 12 months (0–100)	Preprocedure (0–100)	At 12 months (0–100)	Preprocedure (0–100)	At 12 months (0–100)	Preprocedure (0–10)	At 12 months (0–10)
Patient 1	65	35	48	29	61	17	4.0	1.0
Patient 2	50	40	19	29	27	50	5.1	4.7
Patient 3	45	45	5	10	38	33	5.8	5.5
Patient 4	40	10	76	10	44	11	7.5	1.0
Patient 5	74	18	62	0	72	17	9.4	4.5

Abbreviations: IIQ-7, Incontinence Impact Questionnaire short form; UDI-6, Urogenital Distress Inventory short form; UISS, Urinary Inventory Stress test; VAS, visual analog scale.

In Vitro Analyses

Cell Expansion

For the following in vitro analyses, the cells were expanded in vitro in BM consisting of DMEM/F-12 supplemented with 15% human serum (Lonza, Walkersville, MD, http://www.lonza.com) and 1% GlutaMAX (Life Technologies).

Flow Cytometric Surface Marker Expression Analysis

The ASCs (n = 5) at passages 5–6 were analyzed with a fluorescence-activated cell sorter (FACSAria; BD Biosciences, San Diego, CA, http://www.bdbiosciences.com). Antibodies against CD14-PECy7 (BD Biosciences), CD19-PECy5 (BD Biosciences), CD34-APC (Immunotools GmbH, Friesoythe, Germany, http://www.immunotools.de), CD45-PE, CD49d-PE (BD Biosciences), CD73-PE (BD Biosciences), CD90-PE, CD105-PE, HLA-ABC-PE (Immunotools), and HLA-DR-PE (Immunotools) were used. The analysis was performed on 10,000 cells per sample, and unstained cell samples were used to compensate for the background autofluorescence levels. The surface marker expression of $>$2% was defined as a positive expression.

Differentiation Analyses

The myogenic, adipogenic, osteogenic, and chondrogenic differentiation analyses were carried out to verify the multidifferentiation potential of ASCs (n = 5, passages 7–10). All the cultures were maintained for 14 days in differentiation conditions. The BM supplemented with antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin; Life Technologies) served as a control medium.

For myogenic differentiation, the ASCs were plated onto fibronectin-coated (Sanquin, Amsterdam, The Netherlands, http://www.sanquin.nl) wells with a density of 2,631 cells per cm^2. The ASCs were cultured in myogenic medium, containing 5 ng/ml of transforming growth factor β1 (hBA-112; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) in BM for 14 days. The myogenic differentiation was verified by immunostaining using smooth muscle protein 22-α (SM22-α, 1:100; Abcam, Cambridge, U.K., http://www.abcam.com), α smooth muscle actin (α-SMA, 1:100; Abcam), and myosin heavy chain II (MHCII, 1:200; Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com) as primary antibodies. The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) and incubated in primary antibodies. Thereafter, secondary antibodies from goat, donkey, and donkey (1:200, Life Technologies), respectively, were conjugated to primary antibodies. Finally, the cells were mounted with Vectashield (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) and imaged with the fluorescent microscope (Olympus).

The adipogenic, osteogenic, and chondrogenic differentiation have been described in detail previously [13]. Briefly, the adipogenic differentiation was performed by culturing ASCs in adipogenic medium at a density of 2×10^4 cells per cm^2. After 14 days, adipogenesis was verified with Oil Red O staining (Sigma-Aldrich). For the osteogenic differentiation, 2,500 cells per cm^2 were incubated in osteogenic medium, and the osteogenic capacity was studied using alkaline phosphatase (ALP) staining (Sigma-Aldrich). A micromass culture method was used for chondrogenic differentiation, and the chondrogenic potential was verified by Alcian blue staining (Sigma-Aldrich).

Results

Clinical Results

Five patients were followed-up 1 year after the procedure. At 6 months, 1 of 5 patients displayed a negative cough test with full bladder filled with 500 ml of saline. At 1 year, the cough test was negative for three patients, and two of them were satisfied and did not wish further treatment for SUI. There was also improvement according to the 24-hour pad test with the objectively cured patients (Table 2). There were no changes in either urodynamic parameters or in urine residual volume in any of the patients.

There was subjective improvement with all five treated patients according to the UISS, IIQ-7, UDI-6, and VAS (Table 3); however, the first three patients did not indicate improvement on all of the questionnaires. The two patients who benefitted from the ASC treatment were consistent in their answers and were subjectively cured or improved according to the UISS, IIQ-7, UDI-6, and VAS questionnaires. Three of the patients have been operated on after the 1-year follow-up.

With the exception of small hematomas, there were no adverse events from the adipose tissue collection. There were no major complications (urinary retention, hematuria, or urinary tract infection) after the transurethral injections. One patient displayed mild pollacis and dysuria that resolved spontaneously within a week.

In Vitro Results

After ASC isolation, the cells proliferated rapidly in autologous serum containing medium. The live/dead analysis prior to injection therapy confirmed the viability of ASCs in the Contigen: the majority of cells were viable, and only a few dead cells were detected (Fig. 1).

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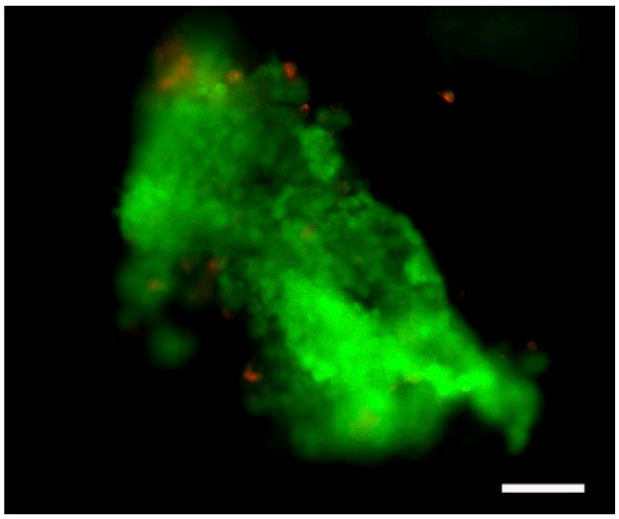

Figure 1. Representative image of viable (green) and dead (red) adipose stem cells mixed with Contigen. Scale bar = 100 μm.

Table 4. Surface marker expression of undifferentiated adipose stem cells analyzed by flow cytometry

Surface Protein	Antigen	Surface marker expression (n = 5)
CD14	Serum lipopolysaccharide-binding protein	41.2 ± 20.0
CD19	B lymphocyte-lineage differentiation antigen	15.9 ± 12.3
CD34	Sialomucin-like adhesion molecule	11.1 ± 7.4
CD45	Leukocyte common antigen	12.8 ± 6.3
CD49d	Integrin α2, VLA-4	83.2 ± 30.6
CD73	Ecto-5′-nucleotidase	99.7 ± 0.5
CD90	Thy-1	98.2 ± 3.2
CD105	SH-2, endoglin	99.9 ± 0.1
HLA-ABC	Major histocompatibility class I antigens	98.8 ± 1.8
HLA-DR	Major histocompatibility class II antigens	2.0 ± 1.5

The data are reported as means ± SD as percentages.
Abbreviations: CD, cluster of differentiation; Thy-1, T cell surface glycoprotein; VLA-4, very late antigen 4.

The surface marker expression analysis of the ASCs showed a positive expression for adhesion molecules CD49d, CD73, and CD105; extracellular matrix protein CD90; and MHC class I isotype HLA-ABC. Minor or moderate expression of markers CD14, CD19, CD34, CD45, and MHC class II isotype HLA-DR were detected, which suggested a low number of cells of hematopoietic and angiogenic lineages (Table 4).

The multidifferentiation potential of ASCs was demonstrated by their capability to differentiate toward myogenic, adipogenic, osteogenic, and chondrogenic cell lineages (Fig. 2). After 14 days of exposing the ASCs to adipogenic medium, the lipid droplets were evident, which confirmed the ASCs potential to differentiate toward adipocytes. The positive ALP staining verified the osteogenic potential of ASCs. The chondrogenic differentiation was detected with Alcian blue staining for cartilage-specific glycosaminoglycans. The ASCs showed positive expression for all the myogenic markers used: SM22-α, α-SMA, and MHCII, thus verifying the myogenic potential of these cells.

Discussion

The purpose of this study was to find out whether transurethral injections of autologous ASCs with collagen could be safe and effective for SUI or MUI treatment. In this study, two of five patients had recurrent SUI, whereas two patients had mixed urinary incontinence.

Two patients had low MUCP, and the BMI of all the patients in the study was ≥25. All of these factors (overweight, mixed incontinence, previous continence surgery, and intrinsic sphincter deficiency) are significant independent predictors for midurethral sling failure [14]. Recurrent incontinence is not an ideal target for any method of treatment. The first three patients did not benefit from the cellular therapy, which may be at least partly due to the introduction of a new method.

Prior to the injection therapy, the adipose tissue was readily collected under local anesthesia. The tissue collection caused some discomfort for the patients, and with the patient who was not eventually treated because of bacterial contamination in repeated samples, there was postoperative hemorrhage that was managed conservatively. The transurethral injections were relatively easily implemented, and the patients were able to return to their everyday activities within the same day. The injections under local anesthesia were well tolerated, and there were no severe complications.

To the best of our knowledge, ASCs have not been previously used clinically for female SUI treatments. However, nowadays stem cell therapies have been under extensive research [15], and prior to this study, some clinical studies on cell-based injection therapies have been published. Mitterberger et al. [16, 17] demonstrated an efficacy of cell-based injection therapy using myoblasts and fibroblasts mixed with Contigen to treat female SUI. However, the primary cells are not the optimal choice because of the difficulties in high-yield expansion of cells. In addition to primary cells, autologous muscle-derived stem cells and allogenic umbilical cord blood stems cells (CBSCs) have both been used in a few clinical trials to treat female SUI patients with promising results [8, 15]. Carr et al. [18] used autologous MDSCs to treat 8 female SUI patients. They demonstrated an improvement of SUI symptoms in 5 patients at the 1-year follow-up, although only 1 patient was totally continent. Furthermore, Sèbe et al. [19] treated 12 female patients with autologous MDSCs, and at 1-year follow-up, 3 patients were dry, and improvement of SUI was detected in 7 patients. Additionally to MDSCs, Lee et al. [20] used allogenic CBSCs in one clinical study to treat 39 female SUI patients. They demonstrated a 36.1% cure rate at the 1-year follow-up. These treatments were demonstrated to be a safe method, and no severe adverse effects were detected. When compared with the aforementioned cell sources, the main advantage of ASCs is that the yield of stem cells from adipose tissue is much higher, and the isolation and expansion of ASCs is also relatively easy [15, 21]. Furthermore, the injection therapy with ASCs was detected as a potential method to treat male urinary incontinence [22], and therefore we wanted to study the efficacy of ASCs for female urinary incontinence. Our approach was to use multipotent autologous ASCs with the bulking agent Contigen for transurethral injection therapy.

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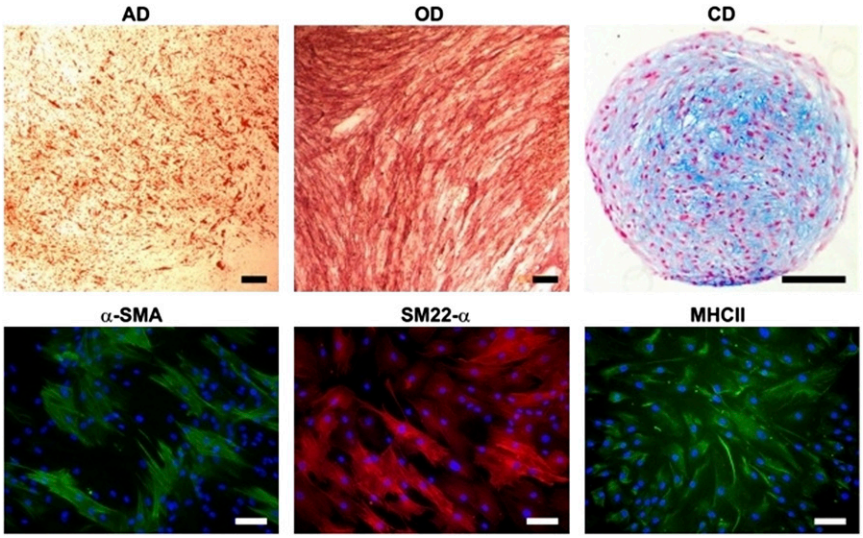

Figure 2. Multipotency of adipose stem cells. AD was detected using Oil Red O staining. The alkaline phosphatase staining ensured the OD, and the Alcian blue staining confirmed the CD. Myogenesis was verified using three immunostaining markers: α-SMA, SM22-α, and MHCII. Black scale bars = 150 μm; white scale bars = 100 μm. Abbreviations: AD, adipogenic differentiation; CD, chondrogenic differentiation; MHCII, myosin heavy chain II; OD, osteogenic differentiation; SM22-α, smooth muscle protein 22-α; α-SMA, α smooth muscle actin.

The mesenchymal origin was confirmed prior to the clinical injections using fluorescence-activated cell sorting analysis after the subsequent cells expansion of ASCs in laboratory facilities. The fact that the analyzed ASCs were at higher passages compared with the ASCs used for injection therapies could have slightly affected the expression results; however, the expression results were concordant with the previous results for ASCs [9, 13, 23]. Compared with the previous studies, the ASCs in our study expressed the hematopoietic markers CD14, CD34, and CD45 slightly more. This difference in expression results could be explained with the patient variation, which is typically high in ASCs [21, 23]. In addition to adipogenic, chondrogenic, and osteogenic differentiation, several studies have exhibited the ASCs' capability to undergo myogenic differentiation [24, 25]. The ASCs used in this study were verified to be multipotent, because they also had a myogenic capacity. Compared with other cell sources, the main advantage of ASCs is that the yield of stem cells from adipose tissue is much higher [15].

Previously, ASCs have been used in several in vivo studies to treat SUI with potential results. Lin et al. [25] showed that transplantation of ASCs via urethral or intravenous injection was an effective treatment and/or prevention of SUI in a preclinical setting in rats. Fu et al. [24] induced rat ASCs to differentiate into myoblasts in vitro prior to transurethral injection therapy and showed that the myoblasts served an important function in improving the urine controlling ability. Furthermore, Wu et al. [26] showed that ASCs transplanted under the urethral mucosa of pudendal nerve-mutilated rats improved maximum bladder capacity, abdominal leak point pressure, maximum urethral closure pressure, and functional urethral length in urodynamic testing compared with the control group.

Although traditionally the ASCs' regenerative capacity was associated with their plasticity, their therapeutic effects appear to be particularly due to their paracrine function through the secretion of a broad range of bioactive molecules. Their potential mechanism of action might be related to immunomodulation, support of growth and differentiation of local stem and progenitor cells, proangiogenic action, chemoattraction, antiscarring, and antiapoptosis effects [27].

New techniques in many tissue regeneration applications are emerging, but midurethral slings are still the first-line surgical treatments for SUI. Reported cure rates of midurethral sling operations rise even up to 90% [28]. Furthermore, other treatment methods such as bulking agents are an option, especially for patients who are unresponsive to traditional treatments or who are not suitable for surgical operation. However, injections with bulking agents have not been as effective as surgical methods, and the main problem has been the relatively poor sustainability of the bulking effect [29]. The short-term subjective cure rate with plain Contigen has been low in women with severe sphincteric incontinence [30]. Several investigators have produced variable results from 23% to 83% cure rates at 1 and 2 years of follow-up after collagen therapy [31].

In our pilot study, we chose bovine collagen (Contigen) to be the carrier of the autologous ASCs, because it is a widely studied biomaterial in SUI injection therapy. Furthermore, it is the only commercially available collagen gel accepted for clinical use. Additionally, collagen is shown to be biocompatible and biodegradable [32]. Our results also showed that ASCs remain viable when combined with Contigen. However, Contigen was not the ideal carrier for cells because of its poor mechanical properties. When Contigen was combined with ASCs, the collagen gel became more liquid, which may have affected the overall bulking efficacy of the injection therapy. More preclinical studies are needed to develop an ideal carrier material for the stem cells.

Conclusion

In this pilot study, we studied the effect of ASCs in combination with injectable bulking agent to treat female SUI. During the 1-year follow-up period, the treatment with autologous ASCs was shown to be safe and well-tolerated and reasonably effective in two of five patients. Tissue engineering techniques hold the potential to provide an efficient treatment for SUI in the

future, but more research is needed to reach this goal. Developing more optimal biomaterial carriers for cells may especially increase the efficiency of the stem cell-based injection therapies for SUI.

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Author Contributions

K.K., R.S., S.H., B.M., E.T., and S.M.: conception and design, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; K.N.: conception and design, administrative support, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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