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NEW APPROACHES TO TREAT WOMEN'S UROGENITAL PROBLEMS

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New approaches to treat women's urogenital problems

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ
الرَّكَّابُ أَحْكَمْتُ بَيْنَهُمْ ثُمَّ قُضِيَ مِنَ الْغَيْبِ خَيْرٌ
سورة هود

The holy Quraan is a (Book) whose verses are perfected and then presented in detail from one who is Wise and Acquainted, Allah, the Lord.

To my parents

My husband

My brother and sisters

ABSTRACT

Women's urogenital problems adversely affect their quality of life. In this thesis, I address two of such urogenital problems: namely, postmenopausal vaginal atrophy (VA) and stress urinary incontinence (SUI).

In the first two studies of this thesis, an intravaginal oxytocin gel (Vagitocin) was used for the treatment of VA in double-blinded randomized controlled trials. The hypothesis was that oxytocin could serve as an alternative to local estrogen treatments for postmenopausal VA, particularly for women, whom estrogens are contraindicated for use by any means, including by local administrations. Breast cancer survivors and other hormone-dependent cancer patients form the core of this group. Oxytocin improved both the subjective symptoms of VA (i.e., dryness, irritation, itching, dyspareunia and postcoital bleeding), including the most bothersome symptom, and the objective features of VA. We observed an increase in the percentage of superficial cells in the vaginal mucosa, an increased maturation value, a decreased vaginal pH and decreased vaginal atrophic scores, as evaluated by a histopathology. Furthermore, oxytocin had no influence on the endometrial lining of the uterus, as evaluated by both ultrasound and endometrial biopsy, and we did not observe any serious side effects. In conclusion, oxytocin induced remarkable clinical and laboratory improvements in postmenopausal VA without inducing any serious adverse effects. Thus, oxytocin (Vagitocin), as a non-estrogenic compound, may be a good alternative treatment for postmenopausal VA, particularly in women who cannot or do not wish to use estrogen-containing products.

Traditional treatments for SUI are not always effective. Therefore, we developed a clinical expansion protocol of mesenchymal stem cells (MSCs). The hypothesis is that MSCs can be injected into the defective sphincter urethrae in order to improve its function by restoring the structure and, eventually, maintaining the continence. We isolated MSCs from the minimally invasive source adipose tissue (Ad) and expanded and stored the MSCs in clinical grade culture and cryopreservation media. We showed that, after isolation and expansion, Ad-MSCs had a stable morphology and shorter population doubling time than standard bone-marrow-derived MSCs (BM-MSCs). Furthermore, Ad-MSCs sustained their surface marker characteristics along five passages and were able to differentiate into both bone and fat lineages. In the last study, we showed that Ad-MSCs could be successfully cryopreserved and thawed in a clinical grade serum- and xeno-free cryoprotectant medium. This is important because such cryopreservation allows Ad-MSCs to be held on the shelf until their planned use(s). The Ad-MSCs exhibited a stable morphology, and they preserved their surface marker characteristics and differentiation potentials into bone and fat lineages. In conclusion, we believe that autologous Ad-MSCs, when cultured and cryopreserved in the described clinical grade media, can be successfully tested for their ability to improve women's SUI in the future.

LIST OF SCIENTIFIC PAPERS

- I. *Al-Saqi SH, Jonasson AF, Uvnäs-Moberg K.*
Oxytocin improves cytological and histological profiles of vaginal atrophy in postmenopausal women.
Submitted manuscript.

- II. *Al-Saqi SH, Uvnäs-Moberg K, Jonasson AF.*
Intravaginally applied oxytocin improves postmenopausal vaginal atrophy.
Under revision in Post Reproductive Health.

- III. *Al-Saqi SH, Saliem M, Asikainen S, Quezada HC, Ekblad A, Hovatta O, Le Blanc K, Jonasson AF, Götherström C.*
Defined serum-free media for *in vitro* expansion of adipose-derived mesenchymal stem cells.
Cytotherapy. 2014 Jul;16(7):915-26.

- IV. *Al-Saqi SH, Saliem M, Quezada HC, Ekblad A, Jonasson AF, Hovatta O, Götherström C.*
Defined serum- and xeno-free cryopreservation of mesenchymal stem cells.
Cell Tissue Bank. 2014 Aug 13. [Epub ahead of print].

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LIST OF ABBREVIATIONS

Ad-MSC	Adipose-derived mesenchymal stem cell
BMI	Body mass index
BM-MSC	Bone-marrow-derived mesenchymal stem cell
Calcein AM	Calcein acetoxymethyl
CB	STEM-CELLBANKER™
CBSC	Cord blood stem cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
E2	Estradiol
EDTA	Ethylenediaminetetra-acetic acid
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
GMP	Good manufacturing practice
H&E	Haematoxlin and eosin
IF	Immunofluorescence
IHC	Immunohistochemistry
ISCT	International society for cellular therapy
IV	Intravenous
LDA	Live/dead assay
LH	Luteinizing hormone
LPP	Leak point pressure
MV	Maturation value
MSC	Mesenchymal stem cell
NAMS	North American Menopause Society
OTR	Oxytocin receptor
QOL	Quality of life
SCR	Screening
SD	Standard deviation
SEM	Standard error of mean

Sk-MSC	Skeletal-muscle-derived mesenchymal stem cell
V	Visit
VA	Vaginal atrophy
VE	Vaginal estrogen
VMI	Vaginal maturation index

1 BACKGROUND

1.1 UROGENITAL PROBLEMS IN WOMEN

This thesis focuses on two urogenital problems in women: vaginal atrophy (VA), also called vulvovaginal atrophy (VVA), and stress urinary incontinence (SUI).

VA is one of the most significant consequences of estrogen deficiency in the menopausal state. Menopause occurs as a result of a reduction in the ovarian function and as a consequence diminished estrogen production for 12 months or more. In the Western world, the average age for entering menopause is 51 years (Greendale, 1999) and it may be genetically determined (Rymer, 2000). The reduction in circulating estrogen concentrations exerts unfavorable effects on the elasticity and collagen synthesis of the vulvovaginal tissue, which adversely influences the growth of the vaginal epithelial lining, causing VA in postmenopausal women. In addition, the reduced circulating estrogen concentration causes atrophy in the urethra, the bladder, the bladder trigone and the vesico-vaginal connective tissues around the bladder neck.

Urinary incontinence (UI) is another major health problem. Though UI may occur at any age, its prevalence increases with aging (Milsom, 2000). SUI is the most common type of UI, indicating an involuntary loss of urine due to a sudden rise in intra-abdominal pressure.

1.2 VAGINAL ATROPHY

The state of the vagina that is characterized by a dry and thin vaginal mucosal wall resulting from a reduction in body estrogen is called vaginal atrophy (VA; or, alternatively, atrophic vaginitis or urogenital atrophy) (Mac Bride, 2010). VA often occurs after menopause. The term VA in clinical practice indicates the presence of a thin, dry and pale vaginal mucosa, as well as the loss of the vaginal wall rugae, the existence of petechiae, the loss of elasticity, friability and vaginal discharge (Sturdee, 2010). The vagina becomes narrower and shortens, resulting in a narrow introitus, especially in the absence of penetrative sex. The cervix may be flushed with the vaginal vault, due to the obliteration of the fornices. The vaginal blood supply reduces to a great extent, and secretions from the sebaceous glands diminish. As a result, vaginal lubrication during sexual stimulation decreases (NASM, 2013). When these changes are accompanied by inflammation, the state is called atrophic vaginitis. A cytological examination of the vaginal mucosa reveals a reduction in the percentage of superficial cells (the most mature cells) and an increase in

primitive cell types, such as parabasal cells.

1.2.1 Anatomy

The vagina consists of a fibromuscular tube, about 8 to 12 cm in length, which connects the internal reproductive organs to the exterior. It extends from the cervix to the vestibule between the labia minora. In the relaxed state, the vaginal walls are normally in apposition. The vagina is H-shaped in its central portion, and the side walls are suspended by their attachment to the lateral paravaginal connective tissue, through which the vagina receives its vasculature (Sokol, 2008). Numerous transverse folds are present in the vaginal mucosal layer, which are called the vaginal rugae (Figure 1).

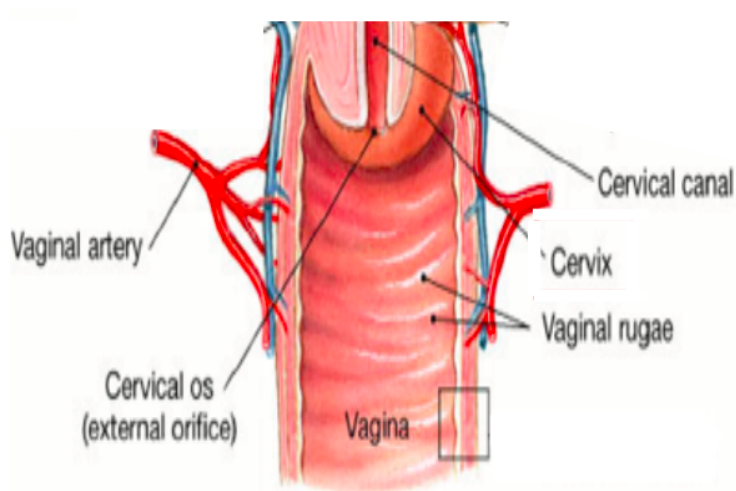


Figure 1: A schematic figure illustrating the vaginal wall and its blood supply. www.creativecommons.org

1.2.2 Histology of the vaginal wall

The vagina is composed of three layers: an inner stratified squamous non-keratinized epithelium, a middle muscular, and an outer fibrous tissue layer (adventitial layer). Under the influence of estrogen (after puberty and before menopause), the vaginal epithelial lining is thick, rugated, lubricated and highly vascularized (Uhlen, 2010).

The vaginal squamous epithelium is composed of three layers. The basal layer is a single cell layer of columnar shaped cells with a uniform and hyperchromatic nuclei and scanty cytoplasm. The immature parabasal layer consists of two to five layers of cuboidal shape cells, with large hyperchromatic nuclei centrally located and comprising about 50 to 75% of the total cell size. The intermediate and superficial layers are of variable thickness. The cells in the intermediate layer are round, with a moderate amount of cytoplasm and small nuclei with dispersed chromatin material. The superficial layer (inner mucosal layer) is the

surface or upper-most layer, consisting of rectangular cells that have small nuclei with chromatin, which comprises about 10 to 20% of the total cell size, and abundant cytoplasm (Helen, 2010; Wylie, 2005) (Figure 2).

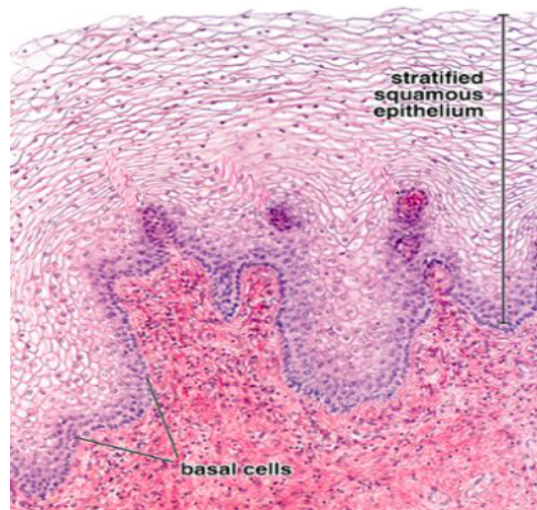


Figure 2: Photomicrograph of the vaginal mucosa showing layers of the vaginal mucosal stratified squamous epithelium www.creativecommons.org

These layers change over the course of a woman's life. Before puberty, the vaginal epithelium consists predominantly of parabasal cells covered with a thin line of intermediate cells. During the reproductive age, the thickness of the vaginal epithelium increases under the influence of estrogen through the addition of multiple layers of mature superficial squamous epithelial cells (Figure 3A). Then, through the cessation of the estrogen effect after menopause, the parabasal cells become dominant again with a reduction in the intermediate and superficial cell numbers (Figure 3B). A schematic illustration of the differences in the cell layers in the pre- and postmenopausal states is shown in Figure 4.

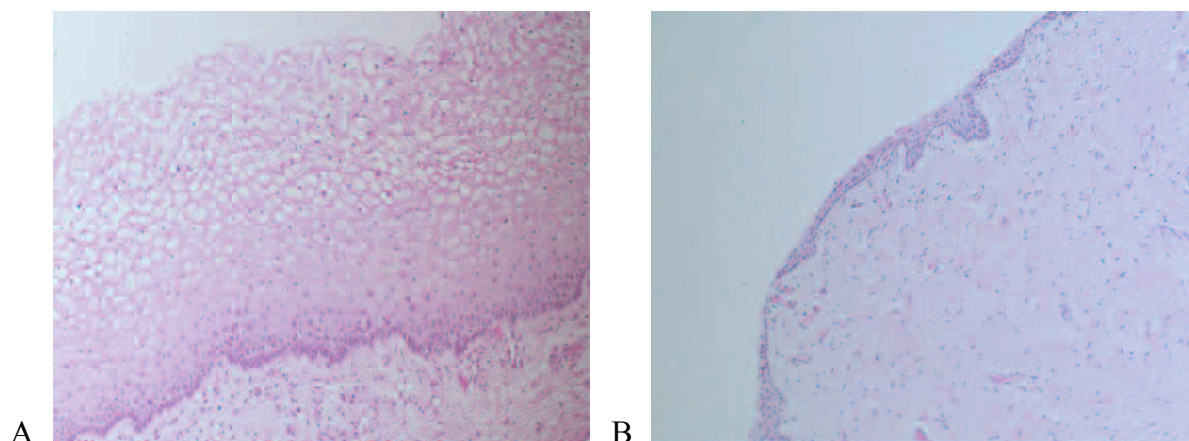


Figure 3: Photomicrograph of the vaginal mucosa. A: Normal vaginal epithelium, B: Atrophied vaginal epithelium. Reprinted with permission from Freedman MA (Freedman, 2008).

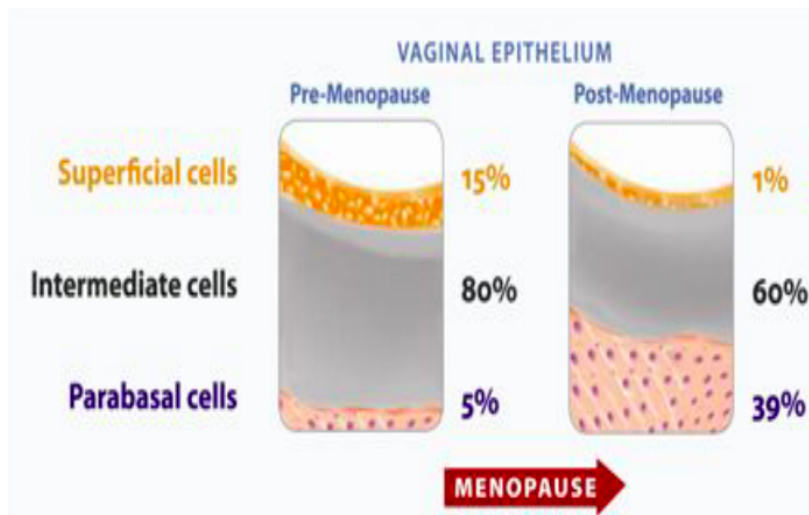


Figure 4: Illustration of the vaginal mucosal layers in pre- and postmenopausal women. www.creativecommons.org

Underneath the epithelium, there is a layer of loose fibrovascular stroma, called the lamina propria, which contains elastic fibers, blood vessels and nerves, but no glands (Wylie, 2005).

The lamina propria sends papillary projections into the epithelial layer. Under normal conditions, there is no keratinization of the vaginal mucosa in either humans or other primates. Therefore, epithelial cell nuclei can be seen throughout the thickness of the epithelium. However, under certain conditions, keratohyalin granules may be present.

The muscular layer (an intermediate layer) consists of two intermingled smooth muscle layers, which may be indistinct. These are the inner circular and outer longitudinal layers. The outer layer is much thicker than the inner one, and it is continuous with the corresponding uterine muscles layer.

Adventitia, the outer layer, consists of the dense connective tissue adjacent to the muscular layer. It contains elastic tissue fibers, which contribute to the strength and elasticity of the vaginal wall. An outer loose connective tissue fuses with the adventitia of the surrounding structures and contains lymphatic and blood vessels, in addition to nerves.

The vaginal wall surface is lubricated by mucous secretion from the cervical, Bartholin's and Skene's glands, as well as by transudate from the blood vessels (Sokol, 2008). There are no glands in the vaginal wall.

1.2.3 Physiology

During menstrual cycles and under the influence of estrogen, the vaginal epithelium undergoes cyclical changes. In the premenopausal state, estradiol concentrations fluctuate based on the time measured in the menstrual cycle. They range between 30 and 40 pg/mL in the early follicular phase to >200 pg/mL at ovulation. While it falls to <20 pg/mL during menopause (Stika, 2010).

VA occurs whenever there is a hypo-estrogenic condition (Mac Bride, 2010). The circulating estrogens in the postmenopausal state are derived mainly from the peripheral conversion of androgens at the adipose tissue level (Judd, 1976; Mac Bride, 2010). Vaginal physiology is regulated by estrogen and estrogen receptors. Two types of estrogen receptors are present: estrogen receptor α , which is present in the vaginal tissue of both pre- and postmenopausal women, and estrogen receptor β , which is either not expressed or expressed to a very low degree in postmenopausal women (Schwartz, 2000). These receptors are expressed throughout the epithelium, the connective tissue and the smooth muscles of the vulva, the vagina, the urethra, the bladder trigone and the vesico-vaginal connective tissues around the bladder neck (Batra, 1987; Blakeman, 2000; Iosif, 1981; Stika, 2010). These receptors are essential for mediating physiological and biochemical functions during the reproductive life (Hodgins, 1998). Estrogen therapy in postmenopausal women appears to be unaffected by the absence of estrogen receptor β (Chen, 1999; Gebhart, 2001). After menopause, there are significant changes in the concentration of pituitary-ovarian hormones. There is a several-fold increase in the follicle stimulating hormone (FSH) and the luteinizing hormone (LH), compared to their levels in the premenopausal period, as well as a reduction in the ovarian steroids of estrogen and progesterone (Burger, 1996) (Figure 5).

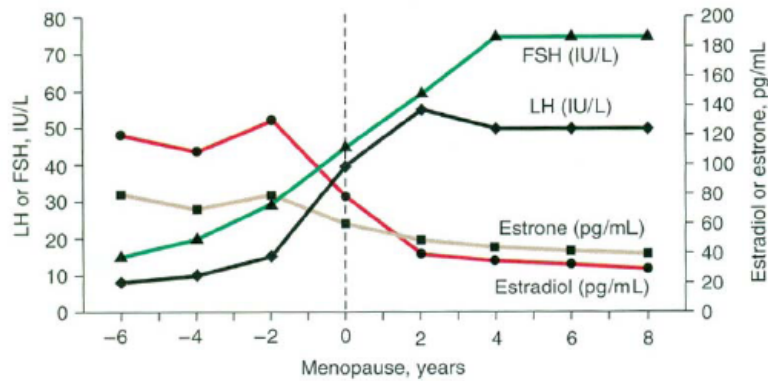


Figure 5: Plasma levels of ovarian and pituitary hormones during the menopausal transition. Estrogens (Estrone and Estradiol), FSH: Follicle stimulating hormone, LH: Luteinizing hormone. www.creativecommons.org

Normally, vaginal epithelial cells exfoliate into the vagina. The exfoliated cells die and release glycogen. This glycogen is synthesized and stored in the vaginal epithelial cells as they migrate toward the surface during the follicular phase. Eventually, the cells are desquamated through a continuous process.

This glycogen hydrolyzes into glucose, which is, in turn, converted to lactic acid by lactobacilli, which form the normal vaginal flora in the reproductive age. The thin epithelial lining of the vaginal mucosa in the postmenopausal state results in lower cell exfoliation into the vagina. The lacking of this cascade results in a rising vaginal pH and a subsequent disappearance of lactobacilli from the vaginal flora. This, in turn, results in an overgrowth of pathogenic bacteria, such as group β streptococcus, staphylococci, coliforms and diphtheroids. These bacteria may induce a symptomatic vaginal infection, which is called atrophic vaginitis (Roy, 2004).

During the reproductive age, vaginal pH varies between 3.8 and 4.5, depending on the action of the lactobacilli, which produce lactic acid and hydrogen peroxide (Roy, 2004). Lactobacilli have an important role in preventing several types of urogenital infections. They prevent the overgrowth of opportunistic and pathogenic microorganisms and, thus, maintain a normal vaginal ecosystem. These infections may include urinary tract infections, bacterial vaginosis and yeast vaginitis (Burton, 2002; Nardis, 2013). In addition, a normal vaginal microbial flora is essential to prevent sexually transmitted diseases, including HIV (Nardis, 2013).

1.2.4 Prevalence of vaginal atrophy

Symptoms of VA affect about 20 to 45% of women in their middle and old ages (Lindau, 2007; Santoro, 2009). VA occurs whenever there is a hypo-estrogenic state. It is more common in the postmenopausal period, which is the time of progressive hypo-estrogenism (Mac Bride, 2010). VA can also occur during other hypo-estrogenic conditions, such as during breast-feeding, after hormonal therapy for breast cancer or after surgical or medical castration, cancer chemotherapy, or pelvic irradiation (Woods, 2012). However, in conditions other than menopause, the atrophic state may resolve itself when there is a restoration of natural estrogen hormone level (Mac Bride, 2010).

1.2.5 Clinical presentation of vaginal atrophy

The reduction in the plasma estrogen concentration in postmenopausal women results in a significant alteration of the lower urogenital tract, including the vulva, the vagina, the cervix, the urethra and the bladder (Ballagh, 2005).

1.2.6 Vaginal symptoms

The most common symptoms of VA are: vaginal dryness, irritation, itching, dyspareunia, post-coital bleeding and vaginal discharge (Mac Bride, 2010). Vaginal symptoms are underreported because, in some countries, not all women seek medical advice. Unlike vasomotor symptoms, VA is a progressive condition, and it is unlikely to resolve spontaneously without intervention. Moreover, it has an obvious effect on woman's quality of life (QOL) and sexual health.

Other conditions, which may have similar symptoms, can be excluded through careful clinical examination. With severe VA, the degree of the vaginal shortening and the narrowing of the introitus may prevent sexual intercourse and preclude the insertion of speculum and vaginal wall inspection. Vaginal secretions of a yellow or brown color discharges may be associated with atrophic vaginitis. Symptoms of VA are not always correlated to the physical findings. In women who are not sexually active, may have few symptoms related to VA. A thorough history and clinical examination are essential to make the correct diagnosis (Mac Bride, 2010).

1.2.6.1 Diagnosis of vaginal atrophy

The diagnosis of vaginal atrophy is a clinical one. However, there are two tests that can be

used to confirm the diagnosis. These are the vaginal pH and the vaginal maturation index (VMI) tests. These determine whether the vaginal pH is elevated to ≥ 4.6 and the proportion of superficial cells in the vaginal smear is $< 5\%$ (Mac Bride, 2010; Roy, 2004). In premenopausal women, the typical proportion of superficial cells is $> 15\%$ (Mac Bride, 2010).

1.2.6.2 Differential diagnoses of vaginal atrophy

Differential diagnoses of VA include any chronic condition that causes vaginal itching, discharge and pain, such as infections, vaginal irritants and vulvovaginal dermatoses. Infections may be caused by viruses, bacteria, fungi or protozoa. The most common vaginal infections are candida vulvovaginitis, bacterial vaginosis, and trichomoniasis. Atrophic changes in the vagina may result in bacterial vaginosis. Chronic vaginal itching may result from irritants such as perfumes, locally applied lubricants or moisturizers and soaps. Similar symptoms may be caused by lichen sclerosus, lichen planus and lichen simplex chronicus (O'Connell, 2008).

1.2.6.3 Risk factors for vaginal atrophy

There are certain factors that may contribute to VA, such as:

Smoking: Smoking increases the likelihood of VA through an indirect effect. It reduces the blood supply to the vagina, resulting in low oxygenation (in the same way that it affects different tissues in the body) (Kalogeraki, 1996). In addition, smoking reduces the effects of the natural body's estrogens, and causing smoking women to experience menopause earlier than non-smokers (Kalogeraki, 1996).

No vaginal births: Women who have never given birth vaginally are more susceptible to VA than those who have had one or more vaginal deliveries (Bachmann, 2000).

No sexual activity: Sexual activity increases blood flow and makes vaginal tissues more elastic. Sexually inactive women are more prone to atrophic vaginal changes (Bachmann, 2000).

1.2.7 Urological symptoms of vaginal atrophy

Healthy genital function is closely connected to healthy urinary system function. VA is usually associated with urinary symptoms. These symptoms may be attributed to recurrent urinary tract infections (UTIs), which are caused by altered normal vaginal flora.

Alternatively, symptoms like frequency, urgency, nocturia or incontinence may occur as a result of the general atrophic process affecting the urogenital system. Daily incontinence episodes in postmenopausal women are twice as common as they are in women in a premenopausal state (Robinson, 2011).

1.2.7.1 Mechanism of urinary symptoms in postmenopausal women

In addition to the altered vaginal flora, which predisposes women to recurrent UTIs, the estrogen reduction in menopause causes an atrophy of the urethral mucosa, a reduction in the collagen content of the surrounding connective tissues, a reduced sensitivity of the urethral smooth muscle to α -adrenergic stimulation and a diminished urethral vasculature pulsatility. These alterations in the lower urinary tract may contribute to the onset of lower urinary tract symptoms, though they are likely to reverse through estrogen replacement therapy (Robinson, 2011; Robinson, 2003; Schreiter, 1976; Tapp, 1986). Intra-vaginal applications of estrogens reduce recurrent UTIs in postmenopausal women by enhancing the growth of the lactobacilli, reducing enteric organisms and, subsequently, reducing recurrent UTI attacks (Ballagh, 2005; Cardozo, 2001). In addition, intra-vaginal estrogen helps to increase the thickness of the urethral epithelial and reduces irritation; however, it does not play any role in the anatomical support of the urethra and bladder neck (Robinson, 2003).

The ageing process reduces the body's natural oxytocin, which adversely affects the entire body's muscle mass (Elabd, 2014). In addition, there is a positive correlation between the plasma estrogen levels and the oxytocin receptor regulation (Gimpl, 2001). Our hypothesis is that these factors may reduce the function of the urethral sphincter.

1.2.8 Current treatment of vaginal atrophy

1.2.8.1 Non-hormonal treatment

Currently, there are various types of over-the-counter non-hormonal preparations for treatment of symptoms related to VA, such as vaginal moisturizers and lubricants. Vaginal moisturizers are water-based preparations and are available in liquid forms, gels or ovules, which are inserted intravaginally every few days. In order to have their optimal effect, these vaginal moisturizers should be used regularly. Vaginal lubricants are either water- or silicon-based and they are short-acting compared to vaginal moisturizers. They are used at the time of sexual activity to overcome the dryness associated with dyspareunia. Vaginal lubricants are safe for long-term use (Mac Bride, 2010).

1.2.8.2 Hormonal treatment

Clinical experience and patient preference are the major determinants for the choice of the hormonal treatment in postmenopausal women (NAMS, 2007; 2013).

1.2.9 Systemic hormone replacement therapy

In 2013, the North American Menopause Society (NAMS) stated that the benefit of systemic estrogen hormones outweigh its risk in women with symptomatic VA who are younger than 60 years or who are in their first 10 years of menopause (NAMS, 2013). When VA is accompanied by other menopausal symptoms, systemic hormone therapy can be used to resolve both. However, systemic estrogens might not be effective for relief from the symptoms caused by VA for 10 to 20% of women (Notelovitz, 1997; Smith, 1993). Therefore, local vaginal estrogen may be added to alleviate vaginal symptoms.

1.2.10 Vaginal estrogen

When vaginal symptoms are a patient's only complaints, local application is the preferred method of hormone delivery. Vaginal estrogen (VE) is used when the non-hormonal measures are not effective. All forms of VE (cream, ring and tablets) are effective at relieving vaginal symptoms, and they are more effective than non-hormonal preparations and placebos (Suckling, 2006). In addition, VE is more effective than oral estrogen for the treatment of VA, relieving vaginal symptoms up to 80 to 90% (compared to 75% in oral estrogen users) (Long, 2006).

1.2.10.1 Adverse effects and safety of local vaginal estrogens

The major issue in VE use is the safety concern regarding its systemic absorption. Several studies comparing types and doses of VE have concluded that there is a limited systemic absorption of VE (Labrie, 2009; Nilsson, 1992; Notelovitz, 2002).

Baseline plasma estradiol concentrations increase from 3 pg/mL to 17 pg/mL following seven days of vaginal application of either a vaginal estradiol tablet or a conjugated estrogen cream (Labrie, 2009). Although this level drops after 14 days of daily VE treatment, it is still statistically significantly higher than the baseline level (Nilsson, 1992). This is important for breast cancer survivors and other estrogen-hormone-dependent cancers (Mac Bride, 2010).

In addition to the local absorption of estrogen into the systemic circulation, it has been shown that VE may be delivered preferentially to the uterus through the uterine first-pass effect

(Alexander, 2004; Bulletti, 1997; De Ziegler, 1997; Tourgeman, 2001). The most important issue regarding the use of estrogen in women who have an intact uterus is the risk of developing endometrial cancer if estrogen is unopposed by progesterone. Data from the Cochrane Database suggests the general safety of low-dose estrogen on the endometrium, but these data come only from short-term studies. Long-term clinical studies are lacking (Suckling, 2006).

Compared to systemic hormone replacement therapy (HRT), low-dose VE is considered to have a lower risk profile. Breast pain, nausea, vulvovaginal candidiasis and vaginal bleeding have been reported following the use of low-dose VE in some trials (Fischer, 2011; Suckling, 2006). There is no increase in the risk for deep venous thrombosis in unsusceptible women; however, data are lacking from women who are at high risk of developing deep venous thrombosis (Suckling, 2006).

1.2.10.2 Vaginal atrophy in breast cancer survivors

The prevalence of symptomatic VA in breast cancer survivors varies from 23 to 61% (Crandall, 2004). The overall survival of breast cancer has increased due to the early detection of the breast cancer through screening programs and to advances in cancer therapy (La Vecchia, 2010). Therefore, it is necessary to ensure vulvovaginal health, especially in the group of women who receive aromatase inhibitors, which worsen VA. Precautions should be taken when prescribing any estrogen product to hormone-dependent breast cancer survivors. The use of VE by breast cancer survivors, even in very low doses, represents a significant concern, due to the potential systemic absorption (Mac Bride, 2010). All treatments used for breast cancer, including surgery, hormonal therapy (such as aromatase inhibitors) and chemotherapy, may exaggerate VA (Cella, 2006; Moegele, 2012; Mourits, 2002). The risk of VA is increased even in breast cancer survivors of premenopausal age, due to the adjuvant therapy used in hormone-dependent tumors (Mortimer, 1999). Therefore, using estrogens in this group of women carries the risk of activating an old, treated, hormone-dependent cancer. In addition, studies have proven that the risk of developing new breast cancers significantly increases in breast cancer survivor women using HRT (Holmberg, 2008).

1.2.11 The vagina as a site for drug delivery

The vagina has the ability to absorb drugs that are often used for local effects to treat local conditions. However, drugs that are used locally (intravaginally) may cause systemic effects, as well (Krause, 2009). The major advantage of using medications vaginally is that

this delivery method overcomes the “hepatic first pass effect”, which adversely affects drug absorption, distribution and elimination. This is advantageous because lower doses than those necessary in oral administration can be used to achieve adequate therapeutic effects. In addition, the frequency of the administration of the medication and the associated fluctuation in circulatory concentrations can be minimized (Alexander, 2004). The vagina is a highly vascular organ, making the epithelial/vascular interface ready for absorption of the locally applied medicine (Krause, 2009). The first uterine pass effect is exerted when the medication is applied to the upper third of the vagina; however, when the medication is applied to the lower third of the vagina, it exerts action mainly on the periurethral region (Cicinelli, 2004).

1.2.12 Oxytocin

The posterior pituitary extract was discovered first for its utero-tonic action in 1906. Oxytocin is a nona-peptide hormone, synthesized in the hypothalamus and secreted to systemic circulation through the posterior pituitary gland (Lippert, 2003). An old study showed that tissue extracts from the thymus, the pineal body and the corpus luteum have the same oxytocin properties by mediating uterine contraction and milk ejection (Ott, 1910). This finding was overlooked until recent years, when studies confirmed that oxytocin is found everywhere in the body and that oxytocin produced peripherally in different organs and in certain types of epithelial and endothelial cells often exerts growth-promoting effects (Chaves, 2013; Gutkowska, 2000).

An *ex vivo* study revealed that oxytocin use in a concentration of 10 μ mol stimulated the growth of vaginal epithelial cells in a rate similar to that of oestrogen (Uvnäs-Moberg and Sjögren, unpublished). In addition, in a previous pilot study, our group has shown that local, intravaginal application of oxytocin increases the number of cell layers in the vaginal mucosal epithelium, from 2 to 3 layers before treatment to 10 to 12 layers after one week of treatment with intravaginal oxytocin 600 IU (Jonasson, 2011).

Different studies have demonstrated oxytocin’s ability to provoke wound healing and alter inflammatory and immune processes in the locally applied area, which might be mediated through a reduction in the locally released inflammatory interleukins (Erkanli Senturk, 2013). Oxytocin increases the survival of skin flaps in rat models, which might be explained through either local modulation of the inflammatory process and/or activation of growth factors (Pettersson, 1998).

Another growth stimulatory mechanism of oxytocin may occur as a result of the increasing of blood supply in the vaginal mucosa and the subsequent transport of oxygen and nutrients to local tissues (Gutkowska, 2000; Petersson, 1998).

Natural aging and oophorectomies can result in a reduction of natural oxytocin secretion, which contributes to the degeneration of the skeletal muscles. This degenerative process of the skeletal muscles has been reversed in a mouse model through systemic oxytocin administration through different signalling pathways (Elabd, 2014). Oxytocin has different types of effects on different cell types. It may promote, inhibit or not interfere with cell proliferation (Cassoni, 2006; Cassoni, 2004). Oxytocin has therapeutic effects on different types of tissues, including gastric mucosal inflammation, colitis (Dusunceli, 2008; Iseri, 2005) and hepatic ischemic injury (Dusunceli, 2008). In addition, oxytocin exerts anti-inflammatory effects on skin oedema and inflammation, an effect mediated through the inhibition of neutrophils-induced oxidative damage (Padhy, 2005). Through mechanisms similar to those mentioned above, oxytocin may activate the growth of the vaginal epithelial cells.

1.2.12.1 Oxytocin receptors

Oxytocin exerts its effects through oxytocin receptors (OTRs), which have been identified in different types of tissues and cells, such as kidney, heart, thymus, pancreas, adipocytes and vaginal epithelial cells (Gimpl, 2001; Uhlen, 2010). In addition, OTRs have been demonstrated in skeletal muscle and muscle satellite cells (muscle-derived mesenchymal stem cells) (Elabd, 2014). These OTRs belong to the rhodopsin-type class I G-protein-coupled superfamily receptor. OTRs are proteins that are located on the cell membrane, nuclear components and the nucleolus and that mediate oxytocin effects in normal and neoplastic cells (Kinsey, 2007). Oxytocin treatment results in an internalization of OTRs into the nucleus. The Western blot test indicates that OTRs on the plasma membrane and in the nucleus have the same biochemical and immunological form (Kinsey, 2007).

Oxytocin exerts variable central and peripheral pharmacological profiles. These profiles are mediated by the presence of different OTR subtypes (Peter, 1995; Verbalis, 1999). Various OTR subtypes exert various physiological functions of oxytocin through the activation of different signalling pathways in different cell types (Rimoldi, 2003; Zhou, 2007). The expression of OTRs in different tissues varies from no expression to mild, moderate and high expression. The vaginal stratified squamous epithelium expresses OTRs in medium range, as identified by immunohistochemistry (Uhlen, 2010) (Figure 6).

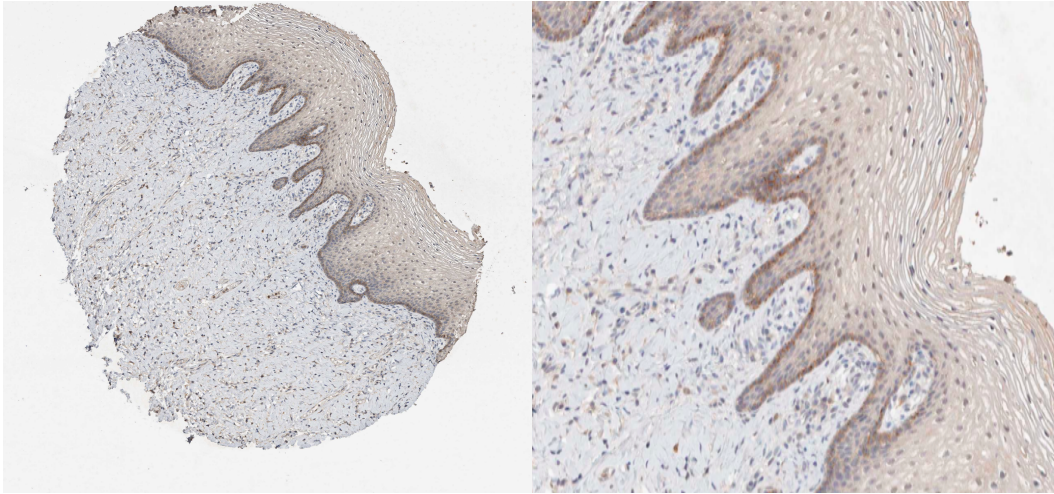


Figure 6: Vaginal stratified squamous epithelium with a moderate expression of oxytocin receptors (indicated as brown staining). Reprinted with permission from The Human Protein Atlas 2010 (www.proteinatlas.org) (Uhlen, 2010).

1.3 URINARY INCONTINECE

Urinary incontinence (UI) is defined as an involuntary loss of urine. It is a chronic health problem that may affect people at any age (Thomas, 1980). It is estimated that UI affects about 200 million people (men and women) worldwide (Furuta, 2007). In women, the prevalence of the condition is about 25 to 45% (Buckley, 2010), which is approximately double that in men (Buckley, 2010), and it increases with age (Milsom, 2000). UI has detrimental effects on both functional and psychological aspects of the QOL (Brazell, 2013). In addition to bearing the economic burden of the problem in terms of incontinence- and caregiver-related expenses, women become more socially isolated. In Sweden and the USA, UI costs represent about 2% of total health care costs (Milsom, 2000). The most common types of UI are stress, urge and mixed incontinence (Davila, 2004).

1.3.1 Stress urinary incontinence

Stress urinary incontinence (SUI) is the most common type and accounts for 50% of UI (Hannestad, 2000; Yoshimura, 2012). It is defined as the involuntary loss of urine due to effort, such as sneezing, coughing, exercise and lifting heavy weights (Davila, 2004). Risk factors for SUI are age, obesity, smoking pregnancy and childbirth (Luber, 2004).

Primarily, the pathophysiology of SUI is confined to two factors: intrinsic sphincter deficiency and urethral hypermobility. Impaired urethral closure due to intrinsic sphincter deficiency is the main mechanism contributing to SUI (Delancey, 2010; DeLancey, 2008; Staskin, 2011; Yoshimura, 2012) (Figure 7).

Treatment options available for SUI include bulking agents, urethral slings, peri-urethral balloons and an artificial sphincter. Severe types of SUI affect life dramatically, and surgery may be the only treatment option for correction. The choice among the four therapeutic options is discussed according to history of the patient, the severity of the condition and the patient's expectations (Boissier, 2013). HRT is not recommended for the treatment of SUI. However, intra-vaginal estrogens might be a useful addition to the treatment of symptoms of an over-active bladder (Davila, 2004). Our special aim with SUI is to develop a minimally invasive method for treatment using cellular therapy. Such a treatment may be used for SUI in both premenopausal and postmenopausal women.

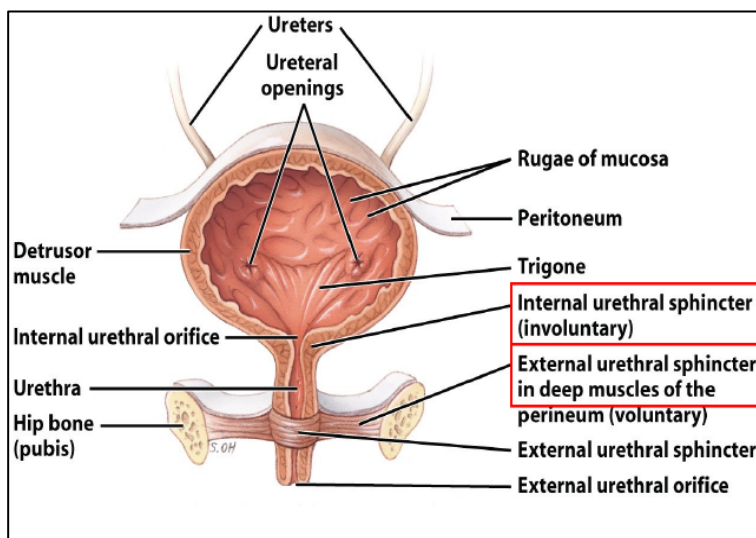


Figure 7: Illustration of the female lower urinary tract with the urethral sphincter complex.
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1.3.2 Regenerative medicine for disorders of the lower urinary tract

The use of regenerative medicinal strategies for the restoration of the urinary tract has not gained as much attention as their use for other areas of medicine. This might be because disorders of the urinary tract, with exception of kidney diseases, are not life threatening, and the available alternative therapies are, to some extent, effective. Therefore, many scientists and physicians have not paid much attention to these disorders. However, due to increases in population aging (WHO Report, 1993) and the associated urological problems, the costs of such disorders have been increasing for society. Cell therapy using a patient's own cells might be an important technology for the treatment of SUI in the future (Sumino, 2013). Mesenchymal stem cells (MSCs) are promising cells for such therapies. MSCs from different sources have been tested in relation to the reparation of the defective urethral sphincter (Carr, 2008; Chancellor, 2000; Kinebuchi, 2010; Yamamoto, 2012; Yiou, 2003).

A summary of some of the preclinical and clinical studies using MSCs for the treatment of SUI is presented in Tables 1 and 2, respectively.

Table 1: Summary of preclinical studies using MSCs for the treatment of SUI.

Model	Cell type	Injection site/ Cell number	Assessment time point	Evaluation	Results	Conclusions	References
Sphincter injury (notexin) in male mice	Autologous Sk-MSC	Periurethral/ 0.25×10^6	1 & 4 weeks	Histology HE	Sphincter regeneration (myofiber formation)	Repair of surgical and obstetric sphincter damage	(Yiou, 2002)
Sphincter urethra in male mice	Satellite cells (myoblast) from striated urethral sphincter	<i>In vitro</i> culture	10 days	IHC	Striated myotube formation expressing α -actinin-2	Possible to use satellite cells for sphincter repair	(Yiou, 2003)
Sciatic nerve transection in rats	Human Sk-MSC	Periurethral/ 1×10^6	4 weeks	LPP/ Histology HE	Increased LPP	Improved physiological outcome in SUI	(Kim, 2007)
Vaginal distension in rats	Autologous Ad-MSC	Periurethral & IV/ 1×10^6	4 weeks	Conscious cystometry & IHC/IF tracking	Higher elastin and smooth muscle content, improved urinary function, increased LPP	Possible to use in treatment or prevention of SUI	(Lin, 2010)

Ad-MSC: Adipose-derived MSC, HE: Hematoxylin and eosin, IF: Immunofluorescence, IHC: Immunohistochemistry, IV: Intravenous, LLP: Leak point pressure, Sk-MSC: Skeletal-muscle-derived MSC.

Table 2: Summary of clinical studies using MSCs for the treatment of SUI.

Model	Cell type	Injection site/ Cell number	Assessment time point	Evaluation	Results	Conclusions	References
39 female patients	Allogeneic CBSC	Transurethral (sub-mucosa of proximal urethra) 400x10 ⁸ /2 ml	1, 3, 12 months	Clinical/ Urodynamic	72% patients >50% improvement after 12 months	Effective treatment for all types SUI	(Lee, 2010)
8 female patients	Autologous Sk-MSC	Periurethral & transurethral/ 4 circumferential injections of 20x10 ⁶ cells	16.5 months	Clinical	5/8 showed improvement, one fully continent	Objective and subjective improvement	(Carr, 2008)
12 female patients	Autologous Sk-MSC	Endourethral/ 10-50x10 ⁶	1, 3, 6, 12 months	Clinical/ Urodynamic	3/12 dry after 12 months and 7 improved by pad test, improved QOL, 2 patients slightly worse	Safe and effective for severe and previously operated SUI	(Sebe, 2011)

CBSC: Cord blood stem cell, Clinical evaluations included: bladder diary, pad weight tests and quality of life measures. Sk-MSC: Skeletal-muscle-derived MSC, QOL: Quality of life.

1.3.3 Mesenchymal stem cells

MSCs were first isolated from bone marrow in 1968 by Friedenstein *et al.* (Friedenstein, 1968). MSCs have also been isolated from many different tissue sources (Barry, 2004; Kode, 2009; Porada, 2006). The international society for cellular therapy (ISCT) has stated the minimal criteria for MSCs as: adherence to plastic; positive for CD105, CD73 and CD90 and negative for hematopoietic and endothelial markers and co-stimulatory molecules CD45, CD34, CD14 or CD11b, CD79 alpha or CD19 and HLA-DR; and the potential for tri-lineage differentiation into bone, fat and cartilage (Dominici, 2006).

1.3.4 Mechanism of action of MSCs

In general, MSCs enhance tissue repair at the transplanted site through their paracrine effects by secreting different cytokines and growth factors (Ortiz, 2003) and by modulating the immune reaction (Hayes, 2012; Reinders, 2010; Wise, 2012). MSCs also have an anti-

inflammatory property, both *in vitro* and *in vivo*, through the secretion of anti-inflammatory cytokines and antimicrobial peptides (Bartholomew, 2002; Le Blanc, 2008; Le Blanc, 2003; Lee, 2011). In addition, cell-cell contact further aids in tissue regeneration (Hayes, 2012). Therefore, both the non-immunogenic and anti-inflammatory effects of MSCs indicate that MSCs are not associated with detrimental early or late effects, such as acute rejection, fibrosis or scar formation, when used in clinical application (Beitnes, 2012).

1.3.5 Adipose-derived MSCs (Ad-MSCs) for the treatment of SUI

In addition to the general regenerative properties of MSCs, the use of autologous Ad-MSCs for the treatment of SUI will solve the issues of allogeneic cell transplantation. Autologous Ad-MSCs may engraft for longer periods of time at the injected site compared to artificial substances, such as collagens, which, today, are used for the treatment of SUI. This is due to the absence of immunological reactions to the injected autologous Ad-MSCs (Furuta, 2007).

Ad-MSCs are capable of long-term proliferation, self-renewal, multilineage mesodermal differentiation and the production of neurotrophins, such as nerve growth factors (Peeraully, 2004), which may help in the repair of the defective sphincter urethrae. In addition, adipose tissue can be obtained in large quantities and through a minimally invasive method under local anesthesia (Furuta, 2007). The availability of MSCs in the adipose tissue is not influenced by age to the same extent as that of MSCs isolated from bone marrow (Casteilla, 2011). Ad-MSCs have shown an *in vitro* ability to differentiate into smooth muscle cells, and such cells have the capability to contract and relax in response to pharmacological stimulation (Ning, 2006; Rodriguez, 2006). In addition, in rat models, Ad-MSCs were able to differentiate into smooth muscle cells when they were injected into the periurethral region (Lin, 2010; Watanabe, 2011), where they improved urethral resistance by increasing leak point pressure (Watanabe, 2011). Furthermore, Ad-MSCs increased the elastin content of the sphincter urethrae of the incontinent rats (Lin, 2010). Therefore, autologous Ad-MSCs have been considered to be a potential minimally invasive tool for the treatment of urinary incontinence (Furuta, 2007).

1.3.6 Clinical applications of MSCs and in urogynecology

Several clinical trials have been performed using MSCs due to the evidence of their tissue repair and anti-inflammatory properties (Gimble, 2007; Zuk, 2001). MSCs have been successfully used in the treatment of bone fractures (Connolly, 1991; Mesimaki, 2009),

ischemic heart disease (Kinnaird, 2004), ischemic stroke (Dharmasaroja, 2009) and graft versus host disease (Le Blanc, 2008). So far, many patients have been treated with MSCs without having any serious side effects (Lee, 2011). Ad-MSCs have been tried as an adjuvant to fat grafts in hemi-facial atrophy (Parry-Romberg disease), where they enhanced graft survival and produced better results than fat grafts alone (Koh, 2012). The action is mediated through the Ad-MSC's paracrine effect (Koh, 2012).

MSCs have been tested in gynecology for the repair of pelvic floor disorders, such as SUI (Lane, 2012). So far, all of the clinical trials performed using MSCs for the treatment of SUI have shown at least partial improvement. A trial using autologous skeletal-muscle-derived MSCs injected into the sphincter urethrae through transurethral and periurethral approaches showed a reduction in incontinence episodes and an improvement in the QOL (Carr, 2008). In a study of umbilical cord blood stem cells in women with SUI, more than 70% of the patients saw about 50% improvement in their urodynamic evaluations after 12 months (Lee, 2010).

1.3.7 Criteria for MSCs used in clinics

There are regulations regarding the clinical use of cell therapies, including MSCs. The good manufacturing practice (GMP) quality system is required by the tissue acts (Burger, 2003; European Union Directive, 2004; National Cell Therapy Group/ Sweden, 2010). Ideally, a chemically defined xeno-free medium should be used. There are many factors that may impact cell culturing. Among these are the culture media and their supplements, the coating of culture surfaces, dissociation enzymes, cryopreservation, oxygen saturation and cell plating density, to mention some. However, the culture medium used for the isolation and expansion of cells is the most central factor. Cells are greatly influenced by the components of the culture medium (Sensebe, 2009). Most of the culture media used for *ex vivo* MSC cultures contain fetal bovine serum (FBS), which harbors the risk of transferring animal-borne infections. In addition, the FBS is engulfed by the MSCs and might be a cause for immune rejection upon transplantation (Horwitz, 2002; Spees, 2004; Sundin, 2007). Moreover, the serum is not a defined supplement and has high batch-to-batch variation (Schallmoser, 2007).

1.3.8 Problems associated with the use of MSCs in clinical applications

Some problems are associated with the use of MSCs in clinical applications, and these must be overcome before MSCs can be routinely used in cell therapy. Among these problems is the

search for the optimal and least invasive collection methods to obtain MSCs in relation to the severity of the condition that requires treatment with MSCs, the proper tissue source for isolation in terms of MSC prevalence, the efficacy and long-term safety of the transplanted MSCs, and the *in vitro* expansion and engraftment of the MSCs.

1.3.9 Isolation and *in vitro* expansion of MSCs

Adipose tissue is an alternative to bone marrow as a source for the isolation of MSCs, since it can be obtained easily and in large volumes (Basu, 2011; Witkowska-Zimny, 2011; Zuk, 2001). Ad-MSCs are similar to MSCs from bone marrow (BM-MSCs) with respect to their phenotype and multilineage differentiation potential (Dicker, 2005). Ad-MSCs are abundant, with each gram of adipose tissue yielding up to 5,000 Ad-MSCs (compared to 100 to 1,000 MSC/ml of bone marrow) (Strem, 2005). Furthermore, Ad-MSCs are present in the adipose tissue regardless of the donor's age (Casteilla, 2011) and have higher proliferative potential than BM-MSCs (Ikegame, 2011). Following the isolation of Ad-MSCs, *in vitro* expansion is needed in order to achieve therapeutic MSC numbers.

1.3.10 Cryopreservation of MSCs

It is critically important to find an effective and clinically approved cryopreservation method to preserve human Ad-MSCs in general and, for potential clinical use, in particular. An optimum cryopreservation system is required to enhance cell expansion and validation for transport between laboratories and clinics and to preserve a stored backup if repeated transplantations are demanded.

The best possible cryoprotectant should be non-toxic and non-immunogenic for both the cells and the recipient patient, preserve maximal cell viability and characteristics, be chemically inert and highly water soluble in low temperatures, induce minimal ice-crystal formation, have a low molecular weight and be affordable (Janz Fde, 2012; Naaldjik, 2012; Pegg, 2007). Different types of cryopreservation solutions to cryopreserve human MSCs have been evaluated. For the time being, dimethyl sulfoxide (DMSO), in combination with a serum, is the most commonly used cryoprotectant.

1.3.11 Studies on oxytocin and MSCs

Recent research has pointed to a connection between MSCs and oxytocin, using both *in vitro* cultures of MSCs with oxytocin and in different animal models, in which oxytocin has been used locally and systemically (Elabd, 2014; Kim, 2012; Kim, 2010; Noiseux, 2012).

Oxytocin is one of the factors that regulates myogenic responses in satellite cells (muscle MSCs) and maintenance of muscle tissues (Elabd, 2014). Therefore, age-related decreases in the oxytocin levels result in reductions in muscle mass (Elabd, 2014). Systemic oxytocin administration in a mouse model enhanced muscle regeneration through the activation and proliferation of the muscle MSCs (Elabd, 2014). Oxytocin pretreatment of umbilical-cord-derived MSCs prior to use for treatment of cardiac ischemia in a rat model showed higher MSC engraftment, reduced cardiac fibrosis and a better cardiac contractility function than was found in the control group (Kim, 2010). Furthermore, oxytocin also induced endothelial cell proliferation and migration (Cattaneo, 2008) and enhanced the anti-inflammatory and anti-fibrotic function of the MSCs (Kim, 2010). In summary, the effects of oxytocin on MSCs include transmigration stimulation, higher integration into the site of transplantation, increased differentiation and an enhancement of the anti-inflammatory and anti-fibrotic effects of MSCs (Danalache, 2007; Kim, 2010). For these reasons, oxytocin might be a good reagent to enhance the therapeutic effects of MSC therapy.

2 AIMS

The aim of this thesis was to find alternative measures for treating women's urogenital problems and to improve their quality of life with minimum adverse effects.

Special concern was made to find alternatives to local estrogen replacement therapy in hormone-dependent cancer survivors' postmenopausal women with vaginal atrophy. In addition, finding a minimally invasive measure for treating women's stress urinary incontinence. To fulfill these aims, four studies were conducted with the following specific aims:

- 1- To investigate an alternative, intravaginal oxytocin, to local estrogen replacement therapy in postmenopausal vaginal atrophy.
- 2- To investigate how intravaginal oxytocin exerts its actions on the atrophied vaginal mucosa with regard to both the subjective symptoms and the objective parameters of vaginal atrophy. In addition, to explore whether intravaginal oxytocin exerts stimulatory effects on the endometrial lining of the uterus or not.
- 3- To determine the minimum effective dose and the optimal treatment regimen of intravaginal oxytocin.
- 4- To optimize isolation and *in vitro* expansion of MSCs from an easily accessible source in a clinical grade medium for future clinical use.
- 5- To optimize a clinical grade cryopreservation system for the storage of Ad-MSCs for future clinical use.

3 MATERIAL AND METHODS

3.1 ETHICAL CONSIDERATIONS

In the first two studies, postmenopausal women with symptoms of VA were recruited through advertisements in the local newspaper. All participants received written and verbal information before they provided written consent to participate in the studies.

The first study was a double-blind phase II-a, placebo-controlled, multi-center trial, conducted at the Karolinska University Hospital Huddinge, Sweden; the Uppsala Academic Hospital, Sweden; and the Northwick Park & St Marks Hospital NHS Trust, United Kingdom.

Ethics approval was obtained from the Regional Ethical Review Board in Stockholm, Sweden (2009/2153-31/1) for the Huddinge and Uppsala sites. The National Research Ethics Service/North London approved the study at the Northwick Park & St Marks Hospital study site (2009-016613-22). In addition, the study was approved by the Swedish Medical Product Agency (151:2010/57995).

The second study was a double-blind phase II-b, placebo-controlled trial, conducted at the Karolinska University Hospital Huddinge. Ethical approval was obtained from the Regional Ethical Review Board in Stockholm, Sweden (2011/1978-31/2). The study was also approved by the Swedish Medical Product Agency (LVFS 2011:19, 2012-02-01).

For studies III and IV, two types of human MSCs were used: Ad-MSCs and BM-MSCs. The studies were approved by the Regional Ethical Review Board in Stockholm, Sweden (Ad-MSCs, approval 2011/1373-31/1 and BM-MSCs approval 446/00 at 2000-12-04). Informed oral and written consent were obtained from all participants. Written and oral informed consent was obtained from the guardians of young bone marrow donors on behalf of the children.

The materials and methods for each study are described in detail in each article or manuscript, and I will briefly discuss them in following sections.

3.2 STUDY I

Postmenopausal women having clinical signs of VA and vaginal cytological smears containing superficial cells <5%, vaginal pH >5, plasma FSH levels >40 IU/L, 17 β -estradiol

levels <70 pmol/L and BMI <29 kg/m² were included. In total, 124 women were screened, 68 women were randomized and 62 women completed the study. All women underwent four visits (V): a screening visit (V1), a randomization visit (V2), a visit two weeks after the start of treatment (V3) and a visit 12 weeks after the start of treatment (V4). Twenty women at the Huddinge site underwent an additional fifth visit for cytological and histological analysis. Endometrial thickness and plasma 17 β -estradiol concentrations were evaluated as a safety measure at V1 and V4.

3.3 STUDY II

Eligible participants were healthy women who were four years postmenopausal (normal or artificial). The women suffered from symptoms of VA and had objective signs of VA, vaginal pH >5 , endometrial thickness <4 mm (as measured by ultrasound), body mass index (BMI) ≤ 30 kg/m², and blood pressure $<150/90$ mmHg. Women with $\geq 5\%$ superficial cells in their vaginal smears, plasma FSH levels <40 IU/L and 17 β -estradiol levels ≥ 70 pmol/l were excluded. In total, 67 women were screened, 64 were randomized and 54 completed the study. All women visited the hospital three times: a screening/randomization visit (V1), a visit two weeks after the start of treatment (V2) and a visit seven weeks after the start of treatment (V3). During each visit, scores of vaginal atrophic symptoms were recorded, the vaginal mucosa was clinically evaluated, the vaginal pH was measured and a smear was collected for cytological investigation. Endometrial thickness was measured by ultrasound, and biopsies were taken in women whose uteruses were intact for the procedure in both V1 and V3. Vaginal biopsies were obtained in V1 and V3 from a subgroup of 24 women for histological analysis.

3.3.1 The gel (studies I and II)

Intravaginal oxytocin (Vagitocin) (Grindex, Riga, Latvia) gel was used in both studies, with different concentrations. The gel was starch-based in study I and hypromellose-based in study II. In both studies, the gel was dispensed to the women in a pre-filled, 1 ml syringe. In study I, the dose of the gel was 600 IU oxytocin or a placebo with a pH of 5.5, administered once daily for two weeks, with a maintenance dosage of twice a week for 10 weeks. However, in study II, we used oxytocin in two different concentrations (400 IU and 100 IU) or a placebo with a pH adjusted to 3.75. The gel was administered once daily over a seven-weeks period. In both studies, the placebo gel was identical to the active gel, except for the absence of oxytocin.

3.3.2 Evaluation of the vaginal pH (study II)

In study II, the vaginal pH was assessed using the pH indicator ECPH601PLUS (Thermo Fisher Scientific). The accuracy of the technique was ± 0.01 units. Vaginal fluid was collected before the vaginal smears were obtained and was applied to the pH indicator for the measurement of pH.

3.3.3 Endometrial thickness (studies I and II)

A vaginal ultrasound examination was performed using the LOGIQ™ P6 ultrasound system (General Electrics, Little Chalfont, Buckinghamshire, United Kingdom) in both study I and study II. Endometrial thickness was measured at the thickest point between the two basal endometrial layers. In study II, an endometrial biopsy was obtained (whenever practical) using an Endorette® endometrial suction curette (Medscand Endorette, E 0020, Medscand Medical AB, Sweden). All the endometrial histology samples were analyzed at Aleris Medilab, Täby, Sweden.

3.3.4 Vaginal cytology (studies I and II)

The proportion between the numbers of the three cell types is referred to as the VMI (Gillespie, 1967; Nilsson, 1995). The VMI is a qualitative measure of the estrogen effect on the vaginal mucosa. A VMI of 0-49 indicates no or little estrogenic effect on the vaginal mucosa, a VMI of 50-64 indicates a moderate estrogenic effect and a VMI of 65-100 indicates a high estrogenic effect. The maturation index, calculated from a cytological smear, represents the proportion of parabasal, intermediate and superficial cells counted in each 100 cells. In the premenopausal period, the MI is parabasal cells=0, intermediate cells=40-70 and superficial cells=30-60, and the variation depends on the cycle phase. In early menopause, the number of parabasal cells increases to 65, that of intermediate cells decreases to 30 and that of superficial cells drops as low as 5. In older women, the parabasal cells might be the only cells in a vaginal cytological smear (Willhite, 2001).

The maturation value (MV) can be determined by multiplying the parabasal cells by 0, the intermediate cells by 0.5 and the superficial cells by 1. The summation of these three figures indicates the MV, and the higher the figure, the more mature the vaginal mucosa (Willhite, 2001). Patient-reported vaginal dryness is not a valid assessment for genital atrophy; rather, the VMI is a better tool for assessment (Greendale, 1999). The VMI also reflects overall hormonal status (Schneider, 1995). Therefore, the VMI is an excellent tool

for evaluating the effect of hormonal treatments on genital atrophy (Stika, 2010).

Two vaginal smears were obtained, one from each lateral vaginal wall, by gentle scratching with the flat, round end of an Ayre's spatula. The samples were placed on a glass slide and immediately immersed in alcohol for fixation. Six different fields of vaginal smears were examined. The percentages of superficial, intermediate and parabasal cells were calculated.

3.3.5 Histology (studies I and II)

A histological assessment of VA was performed by obtaining a vaginal biopsy. Biopsies were taken from the lateral vaginal wall, 2 cm inside the vaginal introitus, under local anaesthetics using Xylocain[®] (Astra Zeneca, 10 mg/ml injection). Biopsies were performed using a 6 mm skin punch from Miltex (York, USA).

In a histological evaluation, the normal vaginal epithelium should consist of 8 to 12 cell layers, containing large, mature cells with abundant glycogen (Figures 2, 3A and 4). Cells undergoing mitosis have a thin parabasal layer, which is composed of 2 to 3 layers of cells. However, vaginal mucosa renders by histopathological evaluation atrophic when the biopsies show a general reduction in the number of cell layers, cells that are small in size due to reduced maturation and a lack of glycogen content (Figures 3B and 4). In addition, the atrophied vaginal mucosa also shows hyperkeratosis and an increased thickness of the basal cell membrane (Jonasson, 2011).

3.3.6 Plasma oxytocin concentrations (study II)

In study II, plasma oxytocin concentrations were measured in 24 women during the screening visit and visit 2 using radioimmunoassay. At visit 2, blood samples were collected 5 minutes prior to the dose administration and then every 30 minutes up to 10 hours after the dose administration.

3.4 STUDIES III AND IV

3.4.1 Sample obtainment (studies III and IV)

MSCs were derived for both studies from five adipose tissue samples obtained from pregnant women who had undergone caesarean sections. Samples were obtained from the subcutaneous fatty tissue from the lower abdomen. BM-MSCs were obtained from bone marrow donors for siblings with blood diseases.

3.4.2 MSC isolation and *in vitro* expansion (studies III and IV)

Ad-MSCs were isolated and expanded for five passages in a serum- and xeno-free medium (Mesencult-XF) (Stem Cell Technologies, Vancouver, Canada) and in Dulbecco's Modified Eagle's Medium (GIBCO, Paisley, UK), supplemented with 10% fetal bovine serum (DMEM-FBS). All cultures were performed in triplicates in both media. BM-MSCs were included as controls. The Mesencult-XF medium is one of the components of the Mesencult-SF kit, which also contains the Mesencult-SF attachment substrate (which is serum-free) and the Mesencult-ACF Dissociation Kit (which is a serum- and xeno-free component optimized for the dissociation and passaging of human MSCs). Trypsin-EDTA was used for dissociation of MSCs cultured in the DMEM-FBS. MSCs morphology, surface marker expression, viability, senescence, population doubling time and differentiation potential towards osteogenic and adipogenic lineages were evaluated.

3.4.3 Cell morphology (studies III and IV)

MSC morphology was determined at each passage using an inverted microscope with an XM10 camera (Olympus IX81, Tokyo, Japan). Cell images were documented using photography. MSC cytoplasmic extension length and width was measured in mm at 10 times magnification for 10 MSCs in three randomly selected images and was quantified with the use of cellSens software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

3.4.4 Beta-Galactosidase staining (studies III and IV)

BM-MSCs culture expanded in the new Mesencult-XF medium showed prominent vacuoles from passage 4, which we thought could be a sign of MSC senescence. Beta-Galactosidase staining was performed at passage 5 using the Senescence Cells Histochemical Staining Kit (Sigma). Cells positive for Beta-Galactosidase stain blue. Under a light microscope, the total cell number and the number of cells stained blue were counted at 10 times magnification. The percent of Beta-Galactosidase-stained MSCs was calculated from five randomly photographed fields.

3.4.5 Population doubling time (studies III and IV)

MSCs were plated at a density of 4×10^3 cell/cm² in triplicates, and the population doubling time (PDT) was calculated at each passage. The MSCs were harvested when they were 70-80% confluent. The PDT was calculated by the equation $PDT = t / ((\log_2 (y/x)))$, where t = the

time from plating to counting the cells, y = the number of cells when counted and x = the initial number of cells.

3.4.6 MSC viability (studies III and IV)

Trypan blue staining was used in both studies to count live and dead cells at initial isolation, as well as the passaging and re-plating of MSCs. Trypan blue is taken up by compromised or damaged cell membranes and thereby stain intracellular compartments. Live/dead assay (LDA), which is a fluorescence-based staining used to determine the ratio of live to dead MSCs immediately post thawing, was also used (study IV). Live cells showed green fluorescence in the cytoplasm due to the conversion of non-fluorescent calceinAM to green-fluorescent calcein following hydrolysis by intracellular esterases. A third method for evaluating MSC viability was the flow cytometry analysis, in which propidium iodide was added to the MSCs immediately before the analysis in both studies. Propidium iodide is a fluorescent dye that binds to the nucleic acid and cannot penetrate intact cell membranes. However, it can penetrate the membranes of dead and dying cells.

3.4.7 Phenotype of MSC (studies III and IV)

Flow cytometry (FACS Calibur, Becton Dickinson, Mountain View, CA, USA) was used to characterize the surface expression for both Ad-MSCs and BM-MSCs. The MSCs were analyzed early (passages 1-2) in studies III and IV, late (passage 5) in freshly propagated MSCs in studies III and IV and after cryopreservation thawing and propagation (up to passage 5) in study IV. The following surface antigens were analyzed using monoclonal FITC or PE conjugated antibodies: CD3, CD14, CD31, CD34, CD45, CD73, CD80, CD90 (Becton Dickinson), CD105 (Endoglin), HLA-I (A, B, C) and HLA-II (DR). Isotype antibodies ($\gamma 1^{\text{FITC}}/\gamma 2^{\text{PE}}$) were used as a negative control.

3.4.8 Differentiation of Ad-MSC and BM-MSC (studies III and IV)

The differentiation ability of the MSCs towards the mesodermal lineages osteoblasts and adipocytes was assayed *in vitro* at passage 5.

3.4.8.1 Adipogenic differentiation (studies III and IV)

Adipogenic differentiation was initiated at 70% cell confluence through a change to an adipogenic induction medium, which consisted of DMEM-high glucose (HG), penicillin, streptomycin, 10% FBS, dexamethasone, indomethacin, 3-isobutyl-1-methylxanthine

(IBMX) and insulin. The adipogenic supportive medium consisted of: DMEM-HG, penicillin, streptomycin, 10% FBS and insulin. The adipogenic medium was replaced every three to four days for three cycles, alternating between inductive and supportive media. MSCs cultured in DMEM-HG, penicillin, streptomycin, and 10% FBS served as a negative control. The MSCs were stained by Oil Red O for the identification of lipid droplets in the cells.

3.4.8.2 Osteogenic differentiation (studies III and IV)

Osteogenic differentiation was initiated at 70% cell confluence through a change to a bone induction medium consisting of: DMEM-FBS, penicillin, streptomycin, dexamethasone, ascorbic acid and glycerophosphate. Negative control MSCs were cultured in DMEM-FBS, penicillin and streptomycin. The medium was changed every three to four days. Alizarin red S and Von Kossa staining were used for the visualization of extracellular calcium deposition.

3.4.9 Cryopreservation of MSC (study IV)

The cryopreservation of MSCs was performed using two different clinical grade cryoprotectant media. The MSCs were harvested from the third passage and cryopreserved using either 10% DMSO in Mesencult-XF or STEM-CELLBANKER™ (CB) (ZENOAQ, Fukushima, Japan). Cold cryoprotectant (4°C) was added directly to the cell pellet, and the cells were transferred on ice to pre-cooled CryoTubes®, then moved directly to -80°C in a polystyrene cryopreservation box. The cooling rate was 1°C per minute. The cells were frozen at a concentration of $0.5-1 \times 10^6$ in 1 ml. After 24 hours, the MSCs were transferred to liquid nitrogen.

3.4.10 Thawing of MSC (study IV)

The MSCs were thawed by incubating the cryotubes in a water bath at 37°C for one to two minutes. The tubes were removed from the water bath while there were still ice crystals in the medium. The cells were transferred to a 15 ml Falcon tube containing 5 ml medium and centrifuged at 300 g for five minutes. Cells that were cryopreserved in CB were suspended in CELLOTION™ (ZENOAQ, Fukushima, Japan) cell washing solution, which is a chemically defined cell washing solution that optimizes cell recovery. The cells that were cryopreserved in DMSO were suspended in Mesencult-XF medium.

4 RESULTS

4.1 STUDIES I AND II

Despite the difference in the dosages and treatment regimens between these two studies, both revealed improvement in the VA of postmenopausal women. In study I, there was a significant increase in the percentage of superficial cells in the vaginal mucosa after two weeks of using intra-vaginal oxytocin (Vagitocin) 600 IU, compared to the placebo. In addition, MV was increased and vaginal atrophic scores were reduced by histology after 12 weeks of treatment with oxytocin 600 IU, compared to placebo.

More improvement in comparison to the placebo was observed in study II, with a daily application of oxytocin for seven weeks in both 100 IU and 400 IU doses. The percentage of superficial cells in the vaginal smears and the MV significantly increased after seven weeks of treatment with oxytocin 400 IU. Vaginal pH and the scores for VA, as evaluated by histology, decreased significantly after seven weeks of treatment with oxytocin 100 IU. Moreover, the most bothersome symptom was significantly reduced after seven weeks of treatment in the women treated with oxytocin 400 IU, when compared with the placebo. About 53% of the women were free of the most bothersome symptom by the end of the trial.

The thickness of the endometrium did not change upon the use of the oxytocin gel, either within or between groups in either study.

4.1.1 Plasma oxytocin concentrations (study II)

The highest plasma oxytocin concentrations were observed at 30 minutes post-dose for both of the oxytocin (Vagitocin) treatment groups. The plasma oxytocin half-life was observed about two hours after dose administration, and it reached a steady state by six hours. For the placebo group, no major variations in oxytocin plasma concentrations were seen at any time point (Figure 8).

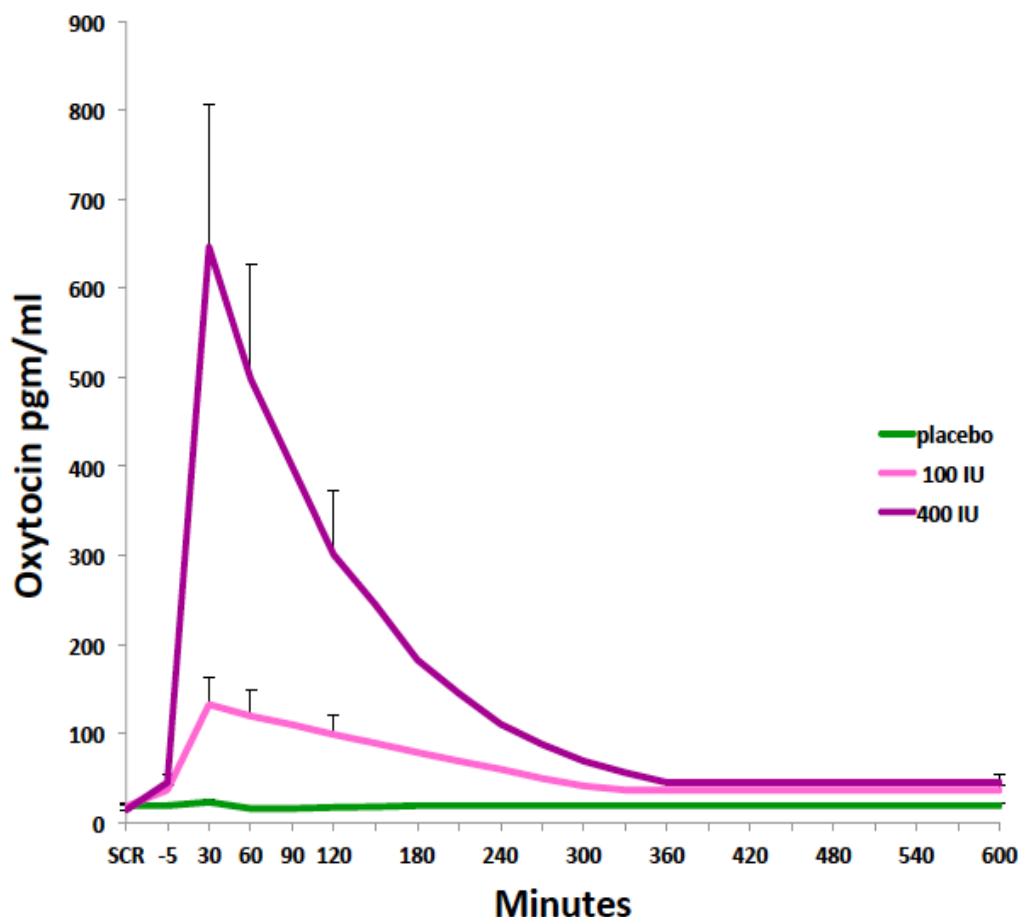


Figure 8: The figure shows the plasma oxytocin concentrations pre-dose at (screening visit and at visit 2), and post-dose at (visit 2) intravaginal oxytocin gel (Vagitocin) administration every 30 minutes up to 10 hours of oxytocin 400 IU (purple), oxytocin 100 IU (pink) and placebo (green). mean±SEM, SCR: screening visit, -5: 5 minutes before oxytocin administration.

The initial absorption of the intravaginal oxytocin into circulation had the same pattern of early burst absorption as the locally used vaginal estrogens. The 17β -estradiol level rises after the vaginal ring insertion ($7.5 \mu\text{g}$ 17β -estradiol), reaching a peak plasma concentration at 30 minutes and reaching a plasma half-life at about 12 hours. After that, a steady state is reached with almost a zero-order release after 24 hours (Hall, 2002) (Figure 9). Vaginal tablets (Vagifem) containing $25 \mu\text{g}$ of estradiol achieve their highest circulating estradiol level after 6 hours and reach their half-life at about 10 hours; then, the curve goes down,

reaching a steady state after 12 hours (Figure 10). After 14 days of Vagifem application, the absorption is marginal; however, it is still above or at the highest postmenopausal level (Novo Nordisk Pharmaceutical Inc., 1998). We have also performed a detailed pharmacokinetic study for intravaginal oxytocin use over two weeks, but the data have not yet been analyzed.

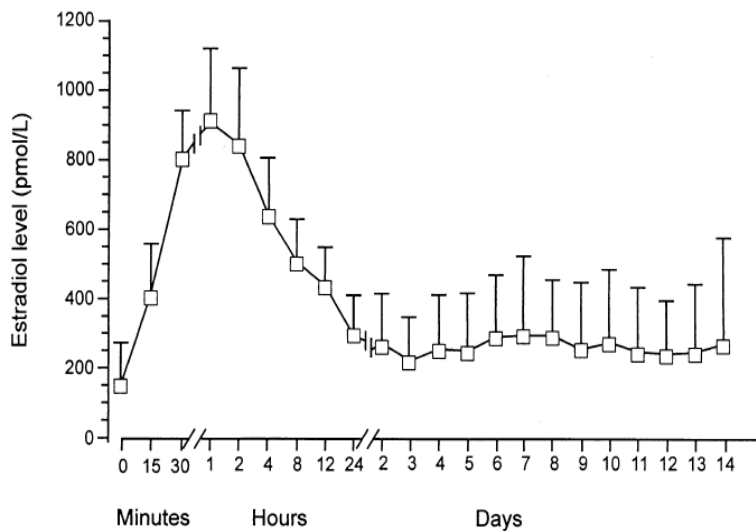


Figure 9: Circulating levels of estradiol (mean±SD) before and after insertion of 7.5 µg Estradiol vaginal ring. Reprinted with permission from the co-author, Britt-Marie Landgren (*Fertility and Sterility*, vol. 78, no. 6, December 2002) (Hall, 2002).

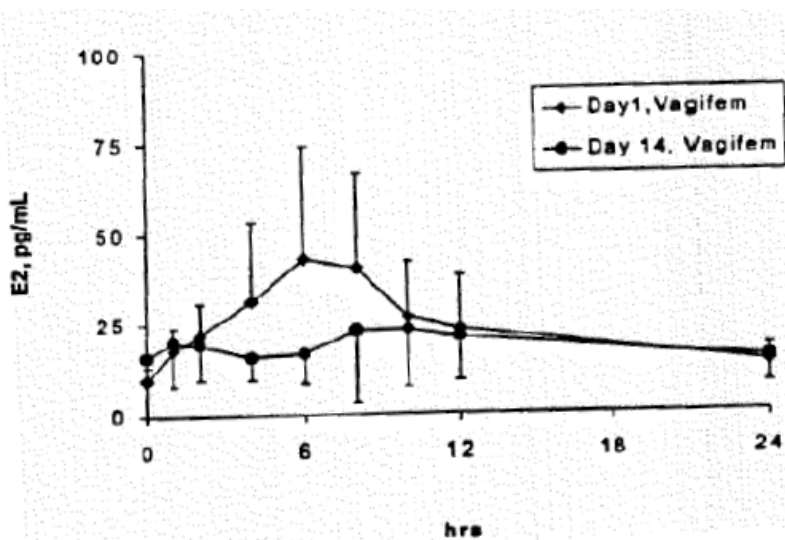


Figure 10: Circulating levels of estradiol after 25 µg Vagifem at day 1 and day 14. E2: Estradiol. www.creareivecommon.org

4.2 STUDIES III AND IV

Ad-MSCs were successfully isolated and expanded in a serum-free culture medium. Ad-MSCs cultured in Mesencult-XF had a shorter population doubling time (33.3 ± 13.7 hours) than those cultured in DMEM-FBS (54.3 ± 41.0 hours, $p < 0.05$). Ad-MSCs cultured in Mesencult-XF displayed a stable morphology and surface marker expression and a higher differentiation potential than Ad-MSCs cultured in DMEM-FBS (study III). Both Ad-MSCs and BM-MSCs were successfully cryopreserved in serum- and xeno-free cryoprotectant media. MSCs cryopreserved in CB or DMSO had morphologies and surface marker expressions similar to those of their respective non-cryopreserved MSCs. The population doubling time of the MSCs cryopreserved in CB and the non-cryopreserved Ad-MSCs was similar, but somewhat higher when cryopreserved in DMSO. The viability of Ad-MSCs was significantly higher after cryopreservation in CB than after cryopreservation in DMSO. Ad-MSCs and BM-MSCs retained their mesodermal differentiation potentials when cryopreserved in both cryoprotectants.

5 DISCUSSION

The mean age of the total world population is increasing, according to the WHO (WHO Report, 1993), and, at the same time, there has been an increase in the attention paid to maintaining normal life styles. Therefore, there is a need to improve health care for women with urogenital problems, which become more common with age and can drastically affect life styles.

In our first two studies, we successfully used intravaginal oxytocin (Vagitoicin) for the treatment of postmenopausal VA. In the first study, we observed significant improvements in the percentage of superficial cells after two weeks of daily application of 600 IU oxytocin. This difference was significant when compared to the placebo. However, the difference was not maintained over the full treatment period of 12 weeks, probably due to a change in the treatment regimen. The vaginal maturation value was significantly improved in the oxytocin group compared to the placebo after 12 weeks. This improvement was achieved, not only by improvement in the oxytocin group, but also by deterioration in the placebo group, which indicates that the effect in the active treatment group was not due to the placebo effect. In addition, the VA scores, as evaluated by histology, were reduced in the oxytocin 600 IU group both over time and in comparison to the placebo after 12 weeks. This disparity in the percentage of superficial cells (by cytology) and in the atrophy scores (by histology) after 12 weeks of treatment could be attributed to the fact that, not only the superficial cell layer, but all of the cell layers are involved in the evaluation of the atrophy scores (by histology). From this study, we concluded that oxytocin is an effective measure for treating postmenopausal VA. However, the dose regimen was not sufficient enough to maintain the increase in the percentage of superficial cells. This regimen of locally applied hormone for VA was successful with estrogens (Chollet, 2011), but not with oxytocin. Therefore, we planned for another study to use intravaginal oxytocin in a daily set in order to achieve the desired clinical and laboratory improvement in the features of VA. At the same time, we sought to determine the minimum effective dose for treating VA without causing serious side effects. Therefore, in the second study, we used intravaginal oxytocin in two doses of 100 IU and 400 IU daily and compared them to the placebo. Both doses were well tolerated, and the women experienced no serious side effects. In this second study, we found a significant increase in the percentage of superficial cells and in the maturation value after seven weeks of daily use of oxytocin 400 IU. However, the reduction in the vaginal pH and the vaginal atrophic scores were clearer in the oxytocin 100 IU group. Moreover, the most bothersome symptom, which is an important variable to investigate in clinical trials, showed highly significant

improvement in the oxytocin 400 IU group in comparison to the placebo after seven weeks of treatment. All of the subjective symptoms of VA were greatly improved in the oxytocin groups, and the most bothersome symptom was no longer experienced by more than half of the women in the oxytocin 400 IU group. In addition, dyspareunia improved almost significantly in the groups that were sexually active. The indirect effect of estrogen on VA was excluded by measuring plasma 17β -estradiol level both before the start and at the end of the trial, which revealed no alteration in the plasma 17β -estradiol concentrations. Interestingly, in either study did oxytocin have no any stimulatory effect on the endometrium, and it was not associated with serious side effects.

The high oxytocin plasma concentrations indicate absorption through the vaginal mucosa, which was dose-dependent. Oxytocin is absorbed into the systemic circulation through the vaginal mucosa in a way similar to that of local estrogen absorption by the initial burst pattern of absorption (Hall, 2002). It reached its peak level rapidly after about 30 minutes and had a half-life of two hours, which is relatively short compared to the half-life of plasma estradiol after vaginal application (Hall, 2002). The longer half-life of vaginal estradiol makes it possible to use this treatment only twice weekly, after an initial period of two weeks of daily application (Nilsson, 1992). On the other hand, the short half-life of oxytocin might have caused the inadequate response in study I, in which oxytocin was used twice weekly over 10 weeks in the second period of the trial.

Since there are no long-term studies on the effect of VE on the endometrium, we cannot judge its safety after prolonged use. The same is true for breast cancer survivors; even a small rise in plasma 17β -estradiol is undesirable in women with breast cancer or other hormone-dependent cancers. Oxytocin applied intravaginally would be a good alternative for treating postmenopausal VA, especially in breast cancer survivors and women with other estrogen-dependent cancers. Further studies using intravaginal oxytocin for longer periods may be necessary to explore its effects on urinary problems, such as SUI and urge urinary incontinence.

Oxytocin exerts its effect on the vaginal epithelium through the oxytocin receptors (Uhlen, 2010). It acts through different pathways to promote cell growth and potentially stimulate the release of local growth factors (Pettersson, 1998; Schaeffer, 1995). In addition, it may play a role in the migration and differentiation of MSCs at the applied site (Cattaneo, 2008; Elabd, 2008; Kim, 2012; Kim, 2010; Noiseux, 2012).

In our third and fourth studies, we optimized clinical grade culture of Ad-MSCs using a

serum-free medium. In order to overcome the problems of immunological rejection of the allogeneic cell transplant, we attempted to optimize the autologous MSCs from a minimally invasive source: namely, adipose tissue. MSCs are also more abundant in adipose tissue than in bone marrow (Strem, 2005). The problem of immune reactions towards animal products, the bovine serum and the associated theoretical risk for prion disease, can be controlled using a clinical grade medium that does not contain any products of animal origin. The Mesencult-XF medium we used was able to preserve the MSC characteristics of the Ad-MSCs in terms of marker expression and mesodermal lineage differentiation (Dominici, 2006). In addition, Ad-MSCs have a much shorter population doubling time in this medium than in the traditional serum-based medium (Ikegame, 2011). By using this medium, we can achieve the number of cells required for transplantation in a shorter period.

Many clinical trials for Ad-MSCs have been registered in different clinical fields, and some of these trials have been completed. Among those, Ad-MSCs have been tested in breast reconstruction, Crohn's disease, spinal cord injuries, fistula and SUI, to mention a few (Lim, 2014).

Because cellular therapy demands the storage of cells for the purposes of transplantation, in addition to the storage of cells if repeated transfusions are entailed, we compared two clinical-grade cryopreservation systems for the cryopreservation of Ad-MSCs. The media were STEMCELL BANKER (CB) and 10% DMSO in Mesencult-XF medium. The CB cryopreservation medium achieved higher MSC viability and preserved better MSC characteristics post-cryopreservation thawing in terms of marker expressions and mesodermal lineage differentiations.

In addition to all of the features of Ad-MSCs mentioned in the literature that made it superior to some other sources of MSCs (Lim, 2014), we had an additional reason for choosing Ad-MSCs: adipocytes harbor OTR, and early precursors of these cells might express OTR in way similar to that expressed in muscle precursor cells (Elabd, 2014). This is an assumption that we need to investigate. The combination of Ad-MSCs and oxytocin might be a solution for several urogenital problems, such as VA and SUI.

Ad-MSCs may be used alone or in combination with oxytocin to treat SUI. Ad-MSCs might be cultured *in vitro* with oxytocin (a study that has already been initiated) before being injecting into the site of the sphincter urethrae. Alternatively, oxytocin can be applied intravaginally (to the lower third of the vagina) following the injection of Ad-MSCs. Another option is to do both Ad-MSCs *in vitro* culture with oxytocin and local intravaginal

application. Finally, oxytocin can be injected directly at the site of the sphincter urethrae, since the intravaginally applied oxytocin gel might not penetrate deep enough to reach the sphincter urethrae in order to achieve therapeutic effects on the urinary symptoms.

6 CONCLUSION AND FUTURE PERSPECTIVES

The aim of this thesis was to find alternative solutions for women's urogenital problems: namely, VA and SUI.

- Oxytocin is a good alternative to local vaginal estrogen preparations in treating signs and symptoms related to VA. It alleviates the most bothersome symptom and increases the vaginal pH. In addition, it helps to improve the laboratory features of VA by increasing the percentage of superficial cells and the maturation value and by reducing histologically determined atrophic scores. Vaginal oxytocin has no effect on the 17β estradiol level; that is, there are no indirect effects of estrogen on the atrophied vaginal mucosa. Furthermore, oxytocin has no stimulatory effects on the endometrial lining of the uterus.
- MSCs were successfully isolated from the adipose tissue and expanded in a serum-free clinical grade medium. The Ad-MSCs had a stable morphology, surface marker expression, shorter population doubling time and the capacity to differentiate into mesodermal lineages. This could be useful in the future, especially in the treatment of women's SUI.
- The Ad-MSCs were successfully cryopreserved in a defined serum- and xeno-free cryoprotectant medium. The Ad-MSCs had potentially higher viability in CB, better preservation of morphology and characteristics in terms of surface marker expressions and mesodermal lineage differentiation.
- We have some ideas concerning the future continuation of this project. In terms of treating SUI, one option might be to precondition the Ad-MSCs using oxytocin *in vitro* before injecting them into the defective site. A study of *in vitro* Ad-MSCs cultured with oxytocin has already been initiated. Another option is to use oxytocin locally as an adjuvant therapy with the injection of Ad-MSCs (whether the Ad-MSCs are preconditioned or not with oxytocin) into the sphincter urethrae. The third possibility is to inject oxytocin alone at the site of the sphincter urethrae. A well-designed clinical study with thorough pre- and post-treatment evaluations and long-term follow up would be necessary to explore the long-term safety and efficacy of such a treatment.
- We hope that, in the near future, SUI can be treated in two outpatient clinical sessions: one to obtain the adipose tissue sample and the second to inject the derived Ad-MSCs into the sphincter urethrae.

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