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Isolation of Adipose Derived Regenerative Cells for the Treatment of Erectile Dysfunction Following Radical Prostatectomy

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Abstract

Stem cells are used in many research areas within regenerative medicine in part because these treatments can be curative rather than symptomatic. Stem cells can be obtained from different tissues and several methods for isolation have been described. The presented method for the isolation of adipose-derived regenerative cells (ADRCs) can be used within many therapeutic areas because the method is a general procedure and, therefore, not limited to erectile dysfunction (ED) therapy. ED is a common and serious side effect to radical prostatectomy (RP) since ED often is not well treated with conventional therapy. Using ADRC's as treatment for ED has attracted great interest due to the initial positive results after a single injection of cells into the corpora cavernosum. The method used for the isolation of ADRC's is a simple, automated process, that is reproducible and ensures a uniform product. Furthermore, the sterility of the isolated product is ensured because the entire process takes place in a closed system. It is important to minimize the risk of contamination and infection since the stem cells are used for injection in humans. The whole procedure can be done within 2.5-3.5 hours and does not require a classified laboratory which eliminates the need for shipping tissue to an off-site. However, the procedure has some limitations since the minimum amount of drained lipoaspirate for the isolation device to function is 100 g.

Introduction

Stem cells have the ability to differentiate into different cell types, and they secrete paracrine factors that are thought to promote the healing process in damaged tissue[1,2,3,4]. They are, therefore, attractive within the field of regenerative medicine, because they can represent a possible curative treatment.

Radical prostatectomy (RP) is the golden curative treatment for patients with low/intermediate risk localized prostate

cancer and a life expectancy > 10 years. The aim of the surgery is eradication of the cancer, but it has several side effects. The prevalence of post-prostatectomy incontinence ranges from 2-60% and erectile dysfunction (ED) is experienced by 20-90% of the patients[5]. Nerve-sparing technique is an option in some patients (Gleason score < 7, low risk of extracapsular disease)[5]. This technique spares the nerves that are responsible for erection, but even though this is possible, many patients still report ED postoperatively.

Treatment options for penile rehabilitation after RP consist primarily of treatment with PDE-5 inhibitors, injection or instillation therapy and vacuum pumps. Medicaments used for penile rehabilitation differ pharmacologically, but their mechanism of action includes relaxation of the smooth muscle cells in the corpus cavernosum. However, many patients experience treatment failure and never achieve an effect of the medicine that enables intercourse[6].

The ED that occurs after RP is thought to be due to structurally irreversible changes[7]. These changes occur in the cavernous tissue and include apoptosis of the smooth muscle- and endothelial cells and fibrosis. The veno-occlusive mechanism that is responsible for a central part of the erection, is impaired by the changes, resulting in poorer filling and hardness of the penis[7].

Many of the patients report that the ED they experience has a negative impact on their quality of life[8]. They are not ready to give up their sexual activity after surgery and, therefore, a curative therapy for ED is attractive, when the other available treatments for penile rehabilitation fail.

In previous trials, including animal and small phase 1 trials on humans, stem cells have shown promising results as an alternative treatment for ED[2,9,10,11,12]. Results show that it is safe to use ADRCs, and that the erectile function is significantly improved after a single injection into the corpora cavernosum[2,9,10,11,12]. Adipose-derived regenerative cells (ADRCs) are thought to support the tissue regeneration by paracrine mechanisms through liberation of multiple hormones, neurotrophic- and other growth factors, cytokines and possibly, micro-RNAs[13]. In addition, ADRCs are able to differentiate into several mature cell-types including endothelial and vascular muscle cells, cartilage cells, osteocytes and neurons[14,15]. These properties make stem cells interesting for developing a permanent new treatment for ED.

Stem cells are divided into several groups, basically those derived from the early embryo (embryonic stem cells) and those from adult tissue (adult stem cells). Adult stem cells include mesenchymal stem cells (MSC) that are multipotent and can be found in the bone marrow, adipose tissue, umbilical cord blood, placenta and dental pulp[17].

Stem cells from the adipose tissue are easy to get access to, unlike stem cells derived from bone marrow. Harvesting stem cells from bone marrow is a risky and painful procedure compared to liposuction. The number of cells possible to harvest from bone marrow will be limited, while only the patient's depots of adipose tissue sets the limit for the number of cells that can be harvested. It is, therefore, possible to isolate a large amount of stem cells from the adipose tissue without a subsequent need to culture the cells to obtain a satisfactory amount. The adipose-derived regenerative cells, also often referred to as the stromal vascular fraction (SFV), is composed of many cell types including MSCs, endothelial cells, pericytes, immune cells and progenitors hereof[18]. These may all play a role in the regenerative process.

The aim of the present study is to investigate the effect of stem cells on ED after RP by using 4 mL of autologous ADRCs isolated from freshly harvested adipose tissue after injection into the corpora cavernosum.

Protocol

All methods described in the protocol were approved by the Danish National Ethics Committee (No. 37054), The Danish Health and Medicines Authority (EUDRA-CT number 2013-004220-11) and the Danish Data Protection Agency (2008-58-0035). The study was registered at ClinicalTrials.gov (NCT02240823). The study was performed in accordance with the Declaration of Helsinki monitored by the Good Clinical Practice (GCP) unit at Odense University Hospital. ADRC preparation was carried out in an authorized tissue establishment for the handling of human tissues and cells at Odense University Hospital (Danish Health and Medicines Authority, Authorization no. 29035).

1. Recruitment of patient/participants

1. To participate in the trial recruit patients who fulfil the following criteria of inclusion.
    1. Recruit patients that are over 18 years of age, sexually active before RP and not infected with the sexually transmitted diseases e.g., human immunodeficiency virus infection, syphilis, or hepatitis.
    2. Ensure that they suffer from erectile dysfunction after a RP which was performed due to prostate cancer.
    NOTE: In this study, the patients were enrolled into the study 5-18 months after the RP and were included regardless of the surgery method: open/ robotic assisted or nervesparing/non-nervesparing.
    3. Ensure that the value of prostate-specific antigen (PSA) had to be undetectable at the clinical follow up after the RP.
    4. Ensure that patients were sexually active before the RP, and still expressing a wish to remain sexually active after the RP. Ensure that pharmacological intervention with a phosphodiesterase type 5 inhibitor (*PDE5* inhibitor) or a synthetic *analog* of prostaglandin E1 (*PGE1*) have been tried before participating and deemed insufficient.
    5. Furthermore, ensure that the patients have enough subcutaneous fat on the abdomen or thigh.
2. Exclude patients from the trial, if there were severe events under the anesthesia during the RP or if they were treated with anticoagulants.

2. Liposuction

NOTE: Liposuction is an operation that removes fat or lipocytes from the subcutaneous area. This part of the protocol is performed as a standard liposuction and the procedure is performed under sterile conditions in an operating theater. All instruments used under the procedure must be sterile and the surgeon must wear scrubs, sterile surgical gown, sterile gloves, surgical mask and hat.

1. Anesthetize the patient.
    1. Ensure that the patient is under general anesthesia during the procedure, and this part is performed by an anesthesiologist.
    2. Disinfect the skin of the abdomen and scrotum with chlorhexidine 0.5% (clorhexidine spirit, 96% ethanol

medicated chlorhexidine digluconate 77% W/W = 83% v/v). Use a surgical marker to draw up the areas on the abdomen were the liposuction is going to be performed. The area will usually be the area between the symphysis and the umbilicus.

2. Make two 6 mm wide incisions in the skin of the abdomen with a scalpel (number 11). Place the incisions symmetrically and laterally from the updrawn area of liposuction.
    1. Put a bit of lubricant such petroleum jelly on the incisions, to make sure that the infiltration cannula slides in and out of the incisions easily during the liposuction.
3. Introduce a size 14 G infiltration cannula through the incisions in the skin. Inject modified Kleins solution subcutaneously in the marked areas parallel to the skin surface. Inject so much of the solution that the targeted tissue becomes swollen and firm, or tumescent.
    NOTE: The modified Kleins solution consists of a solution of 1,000 mL Ringers lactate and 1 mg adrenalin (epinephrine (1:1,000,000)). Do not ass local anesthesia to the solution, as it may have a negative impact on the ADRCs.
    1. Inject a volume of modified Kleins solution that corresponds to the ratio 1:1 to adipose tissue.
    2. Wait for 10 min to maximize the effect of adrenalin and reduce the amount of blood in harvested adipose tissue.
4. Perform a standard liposuction using a jet infusion liposuction to harvest 200-300 mL of adipose tissue. Use blunt cannulas that are hollow, to which an infusion tube and a nozzle are integrated. Attach the cannula to a suction device for liposuction.
    NOTE: A continuous fan-shaped water infusion loosens the fat tissue into fragments that can be easily be suctioned out through the opening in the cannula.
    1. Introduce a 3 mm syringe with a blunt tip through the incisions on the abdomen
5. Collect the adipose tissue in a lipo-collector to preserve the lipoaspirate in a sterile environment. The lipoaspirate will start to separate from the water phase.
6. Use 50 mL sterile syringes to suck up the lipoaspirate from the lipo-collector.
    1. Minimize the volume of water sucked up in the syringes and screw on a plug on the tip of the syringes to keep the adipose tissue sterile.
7. Place syringes with tip downwards in a sterile plastic bag to start the separation of lipoaspirate from the water phase. Place bag in sterile container still with the tips facing downwards to continue separation.
    1. Cover the container with sterile drape to secure the sterile environment during transportation.
        NOTE: According to the user manual of the machine used for the isolation, the lipoaspirate can be stored for up to a maximum of 4 h before isolation of the ADRCs however, we always process it immediately.
8. Close the skin incisions with the surgeons preferred suture material. Place a compression garment, an abdominal binder, around the abdomen to reduce the postoperative edema.
9. Make a penile block by injecting 20 mL bupivacaine 5 mg/mL containing 5 µg of adrenaline.
    1. Use a 20 mL syringe and apply with a 23 G needle, 1 ¼ inches long.

2. Apply 5 mL of bupivacaine in each quadrant in the subcutis.

3. Inject the bupivacaine at two sites, one placed ventrally the other dorsally. Introduce the needle in the whole of its length in the subcutaneous tissue pointing the needle laterally to the right and inject the anesthesia as the needle is retracted. Repeat the injection pointing the needle to the left.
   NOTE: The patient can now be awakened from the general anesthesia by the anesthesiologist.

3. Isolation of ADRCs

NOTE: The isolation process of ADRCs is performed as described in detail in the user manual following the device (see **Table of Materials**). It is important that the procedure is performed under sterile conditions to ensure that the lipoaspirate is not exposed to any contaminants during the purification of the ADRCs. The time taken for the isolation of ADRCs depends of the volume of lipoaspirate, but the whole procedure will approximately take 2.5 h using the semi-automatic device as described here.

1. Place all consumables and enzymes aseptically on a table covered by a sterile disposable surgical towel.
   NOTE: A procedure kit contains all necessary consumables and enzymes needed for the isolation of ADRCs from one patient. Besides this, three 37-39 ˚C, one-liter infusion bags of Lactated Ringers must be available along with sterile clothing, gloves and towels and ethanol for disinfection. Note down all lot numbers of consumables and enzymes and also the temperature of the Lactated Ringers, when connected to the system.
   1. Wipe down the device with ethanol and following the manufacturer's instructions
   2. Load the consumable set onto the device and connect a bag of Lactated Ringers to the system.
   3. Perform the series of semi-automatic tests (system check and leak test) before adding the lipoaspirate.
2. Let the lipoaspirate stand in 50 mL tubes while the steps in 3.1 are performed. This will allow the fat to separate from the liquid phase. Note down the total amount (in mL) of fat tissue and use this to ensure that the amount of loaded fat will be in the range of the device capacity.
3. Load the tissue when prompted to by the device. The machine will now drain off excess fluid and weigh the amount of fat tissue loaded before it is washed with Lactated Ringers.
   1. When the tissue has been washed and drained again, connect a new infusion bag with 37-39 ˚C Lactated Ringers when requested by the machine.
   2. Reconstitute the commercially available enzyme containing collagenase and protease blend in 5 mL Lactated Ringers (one vial of enzyme is enough for fat amounts up to 270 mL).
   3. Ensure that the device displays the amount of enzyme (based on the tissue weight) to inject into the canister with the fat tissue. Once injected, the enzymatic digestion that is carried out under agitation will last around 20 min.
4. After tissue digestion, let the agitator stop and check that the content is separated into two phases: an upper lipid-containing, yellow phase and a lower pink layer that contains the ADRCs. Let the latter layer drained into the cell processing chamber (that goes into the device built-in centrifuge) of the consumable set, while leaving the lipid layer behind.

5. Let ADRCs concentrate in the cell processing chamber during multiple rounds of centrifugation. Now add 10 mL of Intravase (contains DNase, that helps to avoid clumping in the final ADRC suspension) reconstituted in Lactated Ringers. The enzymatic reaction lasts for 10 min after which the ADRCs are washed.
   NOTE: This step is fully automatic, and the machine will inform when the process is done.
6. When the isolation of ADRCs is completed, resuspend the cells in 5 mL of Ringer Lactate. Aspirate the solution into a 5 mL syringe.
7. Mount a 3-way stopcock female Luer-lock on the 5 mL syringe containing the ADRCs, and aspirate 4 mL of ADRCs into another 5 mL sterile syringe.
8. Transfer the last 1 mL into a 1 mL syringe. Use this 1 mL for ADRC characterization e.g., cell count, cell viability, analysis of surface markers by flow cytometry and ADRC differentiation ability.
9. Put on a 25 G needle on the 5 mL syringe containing the 4 mL of ADRC's before it is packed in a sterile drape. Use the ADRCs for injection into the recipient. In this case, the solution of ADRCs will on average contain 8.4-32.7 million cells.

4. Implantation of the ADRCs into corpora cavernosum

NOTE: This is a sterile procedure. All instruments must be sterile, and the person injecting the solution containing the stem cells into the corpora cavernosum must wear sterile gloves. The patient is awake during this procedure and will receive his own stem cells. The ADRCs are injected without counting the cells prior to injection.

1. Keep the solution of ADRCs homogeneous by tilting the syringe gently until it is injected into the corpora cavernosum.
2. Place an aperture drape over the penis. Place a tourniquet at the root of the penis by using a silicone vessel loop. Tighten the loop and secure it with forceps curved pean. Make the tourniquet tight enough to stop the blood flow from the penis.
3. Use two antiseptic swaps to disinfect the penile skin at injection site (lateral site of corpora cavernosum). Inject 1 mL of the solution containing ADRCs, in a direct angle into the corpora cavernosum on the right side at two different places, repeat this step afterwards in the left corpora cavernosum. Total volume of injected ADRCs is 4 mL.
4. Wait for 30 min and then remove the tourniquet.
5. Discharge the patient after 2 h of observation after the injection of ADRCs, to secure that the patient is well recovered following the anesthesia.

5. Postoperative care

1. Recommend the patient to not do any physical performance that can raise the blood pressure within the first week, to prevent development of hematomas.
2. Recommend the patient to wear the abdominal binder for 24 h the first 14 days after the surgery and hereafter for 14 days during the daytime.
3. Treat postsurgical pain with oral acetaminophen (e.g., paracetamol; 1,000 mg, 4 times a day) and oral anti-inflammatory (e.g., ibuprofen; 400 mg 3 times a day).

Representative Results

The presented procedure has been used for an open-label Phase 1 clinical trial including 21 patients[19]. The primary endpoint of the trial was safety of the use of ADRC's in humans and secondary endpoint was the effect of ADRC's on the erectile function.

Twenty-one men were included in the trial with a mean age of 60 years (range 46-69) and a normal erection and active sex life before the RP due to prostate cancer. They all suffered from ED after the RP, with no signs of recovery from medicine available for penile rehabilitation. Six men suffered from incontinence as a side effect to the RP. All men received a single intracavernous injection of ADRC's isolated with the presented method.

All 21 men were followed up with 4 visits in the outpatient clinic at 1,3,6 and 12 months after the injection. The sexual function was evaluated with validated questionnaires – the International Index of Erectile Function-5 (IIEF-5) and Erection Hardness Score (EHS) (Attached as appendices).

No serious events occurred during the in the time of observation. Eight men reported transient redness and swelling at the injection sites, three reported reaction in the penile area. Eight experienced reversible minor events related to the liposuction were reported such as light abdominal discomfort and minor abdominal hematomas. No patients reported discomfort at the 1 month follow up. Eight out of fifteen (53%) in the continent group reported the erectile function were sufficient for intercourse at 12 months. The group of 6 incontinent men did not show any improvement of erectile function.

	Continent	Incontinent
Included patients	15	6
Significant effect	8	0

Table 1: The table shows the differences in significant effect of the treatment between continent and incontinent patients.

Discussion

The presented procedure for isolating ADRCs is not limited to be used only for ED therapy, but can be used in multiple other forms of treatment and experiments. Our trial showed that autologous, freshly isolated ADRCs are safe to use, and the treatment is well tolerable in a 12 month follow up.

Before the procedure is used there are some considerations to be made. A disadvantage of this procedure is that the patient must be under general anesthesia during the liposuction. Liposuction is possible to perform under local anesthesia, but a previous trial has shown that a combination of local anesthetics and adrenaline may have a negative impact on cell growth of fibroblasts[20]. The risk of being under general anesthesia is generally low, but a negative outcome is still seen. This risk must be taken in mind when patients are selected for the treatment. Liposuction is a surgical procedure and will, therefore, always carry at risk of complications. As with all other surgical procedures, there is a risk of post-operative bleeding resulting in formation of

hematomas and a risk of infection. Some of our patients did also report a transient reduced sensitivity of the abdominal skin. An immediate complication to liposuction is bleeding. It is known that patients risk getting systemic complications when large amounts of tissue is removed. The liposuction in this procedure was relatively small and therefore not considered a risk factor.

Complications to the injection of cells is reported as transient redness, tenderness, and hematomas.

The device used for the isolation of the ADRCs, was chosen because the system is CE approved. In Denmark, it is mandatory to use CE approved equipment because the authorities categorize treatment with stem cells as a drug testing trial, when the ADRC is used for injection in humans.

The device does have advantages such as the whole procedure being standardized and performed in a closed sterile environment without requiring a highly classified laboratory. The risk of contamination is, therefore, reduced. This ensures that the procedure is uniform and reproducible, and that the quality of the end-product is always the same each time (however, it also depends on the quality of the input tissue). Performing the isolation on the device is easy and does not require specially educated operators.

One limitation of the device is that the minimum input into the machine is 100 g drained lipoaspirate. As the liposuction is completely finished before the cell processing starts (and in our case, at a different location, not in the OR) in our experience, this will require the amount of lipoaspirate to be at least 125 mL using the rough measure of the 50 mL syringes. Otherwise there is a risk that there will not be enough material for the machine to proceed with. Also, using the upper end limit (max input is 425 mL) would result in a very long isolation procedure.

Adult stem cells from both adipose tissue and bone marrow seem to have capacity for self-renewal and differentiation like embryonic stem cells. One of the advantages of ADRCs over haemopoietic stem cells is a 100-500 times higher yield per tissue volume as compared to bone marrow[21,22], and, therefore, ADRCs do not need to be cultured. Furthermore, haemopoietic stem cells are more difficult and more painful to harvest from the patient than adipose tissue. In many situations, adipose tissue is just a waste product after surgery. It is possible to culture stem cells to give a higher yield suitable for angiogenesis, however freshly isolated ADRC's may have higher angiogenic potential than cultured[23].

Disclosures

The author Søren P. Sheikh is CEO at Blue Cell Therapeutics, Copenhagen, Denmark.

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