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Minced tissue in compressed collagen- a cell containing bio-transplant for single-staged reconstructive repairs

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Abstract:	Conventional techniques for cell expansion and transplantation of autologous cells for tissue engineering purposes can take place in specially equipped human cell culture facilities. These methods include isolation of cells in single cell suspension and several laborious and time-consuming events before transplantation back to the patient. Previous studies suggest that the body itself could be used as a bioreactor for cell expansion and regeneration of tissue in order to minimize ex vivo manipulations of tissues and cells before transplanting to the patient. The aim of this study was to demonstrate a method for tissue harvesting, isolation of continuous epithelium, mincing of the epithelium into small pieces and incorporating them into a three-layered biomaterial. The three-layered biomaterial then served as a delivery vehicle, to allow surgical handling, exchange of nutrition across the transplant, and a controlled degradation. The biomaterial consisted of two outer layers of collagen and a core of a mechanically stable and slowly degradable polymer. The minced epithelium was incorporated into one of the collagen layers before transplantation. By mincing the epithelial tissue into small pieces, the pieces could be spread and thereby the propagation of cells was stimulated. After the initial take of the transplants, cell expansion and reorganization could take place and extracellular matrix mature to allow ingrowth of capillaries and nerves and further maturation of the extracellular matrix. The technique minimizes ex vivo manipulations and allows cell harvesting, preparation of autograft, and transplantation to the patient as a simple one-stage intervention. In the future, tissue expansion could be initiated around a 3D mold inside the body itself, according to the specific needs of the patient. Additionally, the technique could be

	performed in an ordinary surgical setting without the need for sophisticated cell culturing facilities.
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TITLE:
Minced Tissue in Compressed Collagen: A Cell-Containing Biotransplant for Single-Staged Reconstructive Repair

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KEYWORDS:
Tissue Engineering, Biomaterial, Tissue Scaffold, Cell Expansion, Bladder, Plastic Compression, Collagen, Minced Tissue, Polymer, Autograft

SHORT ABSTRACT:
Tissue engineering often includes *in vitro* expansion in order to create autografts for tissue regeneration. In this study a method for tissue expansion, regeneration, and reconstruction *in vivo* was developed in order to minimize the processing of cells and biological materials outside the body.

LONG ABSTRACT:
Conventional techniques for cell expansion and transplantation of autologous cells for tissue engineering purposes can take place in specially equipped human cell culture facilities. These methods include isolation of cells in single cell suspension and several laborious and time-consuming events before transplantation back to the patient. Previous studies suggest that the body itself could be used as a bioreactor for cell expansion and regeneration of tissue in order to minimize *ex vivo* manipulations of tissues and cells before transplanting to the patient. The aim of this study was to demonstrate a method for tissue harvesting, isolation of continuous epithelium, mincing of the epithelium into small pieces and incorporating them into a three-layered biomaterial. The three-layered biomaterial then served as a delivery vehicle, to allow surgical handling, exchange of nutrition across the transplant, and a controlled degradation. The biomaterial consisted of two outer layers of collagen and a core of a mechanically stable and slowly degradable polymer. The minced epithelium was incorporated into one of the collagen layers before transplantation. By mincing the epithelial tissue into small pieces, the pieces could be spread and thereby the propagation of cells was stimulated. After the initial take of the transplants, cell expansion and reorganization would take place and extracellular matrix mature to allow ingrowth of capillaries and nerves and further maturation of the extracellular matrix. The technique

minimizes *ex vivo* manipulations and allow cell harvesting, preparation of autograft, and transplantation to the patient as a simple one-stage intervention. In the future, tissue expansion could be initiated around a 3D mold inside the body itself, according to the specific needs of the patient. Additionally, the technique could be performed in an ordinary surgical setting without the need for sophisticated cell culturing facilities.

INTRODUCTION

Most tissue engineering studies on transplantation to the skin and urogenital tract include autologous cell harvests from healthy tissue and cell expansion in specially equipped cell-culturing facilities[1,2].

After cell expansion, cells are usually stored for later use when the patient is prepared to receive the autograft. Nitrogen freezers allow long-term storage at low temperatures of -150°C or lower. The process of freezing must be careful and controlled in order not to lose the cells. One risk of cell death is crystallization of intracellular water during the thawing process, which can lead to rupture of the cell membranes. Cell freezing is usually performed by slow and controlled cooling (-1°C per minute), using a high concentration of cells, fetal bovine serum, and dimethyl sulfoxide. After thawing, the cells need to be processed again by removing the freezing medium and culturing on cell culture plastic or a biomaterial before transplantation back to the patient.

All the above-mentioned steps are time-consuming, laborious, and costly[3]. In addition, all *in vitro* processing of cells intended for patient transplantation are highly regulated and requires well-trained and accredited personnel and laboratories[4]. All in all, to procure a safe and reliable manufacturing process, the technique could only be established in a very small number of technically advanced centers and a wider use in common surgical disorders is doubtful.

In order to overcome the limitations of cell culturing in the laboratory environment, the concept of transplanting minced tissue for cell expansion *in vivo* is introduced by using the body itself as a bioreactor. For these purposes, the autografts would preferentially be transplanted on a 3D mold according to the shape that is needed for the final reconstruction in the organ of interest[5-7].

Originally, the idea of transplanting minced epithelium was presented by Meek in 1956 when he described how epithelium grows from the edges of a wound. He demonstrated that a small piece of skin would increase its margins and thereby its potential for cell expansion by 100% by cutting the piece twice in perpendicular directions (Figure 1)[8]. The theory has been supported by the use of meshed partial thickness skin grafts for skin transplantation[9] and in skin wound healing models[10].

(Place Figure 1 here) (An animated cartoon could be added in the video)

The present study is based on the hypothesis that the same principle could be applied to the subcutaneous tissue by placing minced epithelium around a mold. The epithelial cells would mobilize from minced transplants (reorganize), cover the wound areas (migrate) and divide (expand) in order to form a continuous neoepithelium that covers the wound area and

separates the foreign body (the mold) from the inner body (Figure 2).

(Place Figure 2 here) (An animated cartoon could be added in the video)

Although previous *in vivo* studies show promising results, further improvements could be achieved by reinforcing the autografts so that the regenerated epithelium could withstand mechanical trauma better[7]. For these purposes, important prerequisites for a successful biomaterial were identified, such as: easy diffusion of nutrients and waste products, possibility to mold in a 3D manner and easiness of surgical handling. Conclusions were made that these needs could be met by adding a composite biomaterial to the minced tissue.

The current study aimed at developing a scaffold composed of minced tissue in plastic-compressed collagen containing a reinforcing core of a biodegradable fabric. By these means, viable cells could migrate from the minced tissue particles and proliferate with morphological features characteristic of the original epithelium (skin or urothelium). Using plastic compression, the scaffold was reduced in size from 1 cm to about 420 µm as the minced particles were encased in the upper layer collagen. The core fabric could be any polymer but needs to be modified with a hydrophilic surface in order to interlink with the covering collagen layers[11].

The method provided an enhanced scaffold integrity by incorporating an electrospun mat consisting of PLGA within two plastic compressed collagen gels using it as a scaffold for culturing minced bladder mucosa or minced skin from pigs. The construct was maintained in cell culture conditions for up to 6 weeks *in vitro*, demonstrating successful formation of a stratified, multilayered urothelium or squamous skin epithelium on the top of a well-consolidated hybrid construct. The construct was easy to handle and could be sutured in place for bladder augmentation purposes or covering of skin defects. All parts of the tissue scaffold are FDA-approved and the technique could be used for single-stage procedures by tissue harvesting, mincing, plastic compression, and transplanting back to the patient as a single-staged intervention. The procedure could be performed for tissue expansion and reconstruction under sterile conditions in any general surgery unit.

PROTOCOL

All animal protocols were pre-approved by the Stockholm County Committee on Animals and all procedures conformed to the regulations for animal use, as well as relevant federal statutes.

1) Animal procedures

1.1) Preparing the animal for surgery

1.1.1) Prepare the surgical table with all the materials and instruments needed for the operation under sterile conditions. Perform the surgery exclusively under sterile conditions to reduce the risk of infection and optimize the conditions in survival surgery.

1.1.2) Fast the animal for 12 hours prior to surgery and measure its weight. Administer an intramuscular injection of azaperone (2 mg/kg) for premedication. Anesthetize the animal with intramuscular injections of tiletamine hypochloride (2.5 mg/kg), zolazepam

hypochloride (2.5 mg/kg), medetomidine (25 µg/kg) and atropine (25 µg/kg).

1.1.3) Inject phenobarbiturate (15 mg/kg) prior to endotracheal intubation and continue general anesthesia with 0.8–2% isoflurane. Insert a peripheral vein catheter in one ear and infuse glucose (25 mg/mL) intravenously during the procedure to maintain the well-being of the animal.

1.1.4) Place monitoring devices on the ear or tail to check temperature, blood pressure, saturation, and peripheral pulse. Control anesthesia by pain stimulation of the ears or hooves. Apply ointment to eyes to prevent dryness while under anesthesia.

1.2) Excision of bladder biopsy specimen

1.2.1) Catheterize the bladder using a 10-French silicone catheter by introducing a speculum to view the urethra and insert the catheter through the urethra and into the urinary bladder to empty the urine under semi-sterile conditions. Fill the bladder with sterile saline solution to a pressure of 20–25 cm H_2O, about 100–300 ml, and then empty to 20 cm H_2O (approx. 8 mL/kg of body weight).

1.2.2) With the pig carefully turned to a side position, perform a basic preoperative sterilization of the skin with application of chlorhexidine gluconate. Apply a diathermy plate to the shoulder after removing the pelage with shaving shears.

1.2.3) Turn the pig carefully into a supine position and remove the abdominal pelage using shaving shears and do a basic preoperative sterilization of the abdominal skin with application of chlorhexidine gluconate.

1.2.3.1) Embed the extremities to minimize the risk of hyperextension damage to muscular nerves to the extremities during the supine surgical positioning. Sterilize the abdominal skin of the animal with successive applications of chlorhexidine gluconate and place sterile draping around the surgical field.

1.2.4) Prior to skin incision, apply an intravenous analgesic consisting of buprenorphine (45 µg/kg), carprofen (3 mg/kg) and local injection of lidocaine in the midline below the umbilicus. Check for pain reaction by grasping the skin with forceps. Make a lower midline incision through the fascia and peritoneum using diathermy for control of bleeding. Localize the bladder that has a fully intraperitoneal position in the pig and can be freely exposed by mobilizing it through the surgical wound.

1.2.5) Take hold of the bladder with the forceps and measure it with a sterilized measuring tape and mark an elliptic-shaped biopsy specimen approximately 12 cm longitudinally and 9 cm transversally, using a sterilized pen (the pig tolerates a quarter reduction of bladder size very well). Excise the marked area, using a scalpel, and place the bladder biopsy specimen in DMEM under sterile conditions.

1.2.6) Perform autotransplantation with the biotransplant or close the bladder by suturing it with 5-0 Vicryl in two layers. Close the abdominal fascia carefully with 2-0 or 3-0 running Vicryl. Close the subcutis with 3-0 Vicryl and the skin with 3-0 Ethilon. Place a dressing on

the wound and carefully attend to the animal until it has recovered sufficiently from the anesthesia and does not express pain.

1.2.7) Move the animal to the animal care facility to secure conditions for postoperative care. Put the animal in a single cage with a heating lamp and attend to the animal until full recovery from the anesthesia and then let the animals be stalled in pairs.

1.2.8) Provide an uneventful recovery regarding pain and well-being and administer buprenorphine (45 µg/kg) intramuscularly for postoperative analgesia and trimetoprim (4 mg/kg) and sulfonamide (20 mg/kg) twice daily for three days and once daily for five days to reduce the risk of postoperative infections.

1.3) Excision of skin biopsy specimen

1.3.1) Prepare the surgical table with all the necessary materials. Anesthetize the animal as previously described in 1.1. Remove the pelage using wax, wash and sterilize the incision area with betadine and 70% alcohol and then place sterile draping around the incision area.

1.3.2) Use a dermatome to harvest a 0.3 mm partial thickness skin biopsy specimen. Place the skin specimen in DMEM before mincing, as described in 2.2. Cover the wound area with fatty ointment and a dressing.

2) Minced tissue preparation

2.1) Bladder mucosa

2.1.1) Wash the bladder biopsy twice in DMEM. Place the bladder biopsy specimen onto a sterilized dissecting plate with the mucosa facing upwards and fix one of the sides to the plate using dissection pins.

2.1.2) Separate the mucosal tissue from the detrusor muscle using fine scissors and forceps (Figure 3) and keep the mucosa moist by dripping saline or DMEM over it.

2.1.3) Use the mincing device by placing it on the mucosa and then pass the device from one end to the other vertically and horizontally, applying manual pressure to obtain pieces of minced tissue of 0.8x0.8 mm (0.8 mm is the distance between the rotating cutting blades).

2.2) Skin

2.2.1) Place the skin onto a sterile dissecting plate and use surgical scissors to separate the epidermis from subcutaneous fat and dermis. The epidermis is thin and translucent (approx. 0.3 mm) when it is ready for mincing.

2.2.2) Use the mincing device by placing it on the epidermis. With pressure, pass the device from one end to the other vertically and horizontally to obtain pieces of minced tissue of 0.8x0.8 mm.

3) Preparation of plastic compressed PCL/collagen autografts

3.1.1) Place all ingredients on ice to keep cold. Utensils needed: Falcon tube, 10X DMEM, 1X DMEM, 1N NaOH and rat tail collagen type 1.

3.1.2) Mix 2 mL of 10X DMEM (carefully to avoid bubbles) with 12 mL of collagen type 1. Add 1N NaOH, drop by drop, to bring pH up to 7.4–8 (color in the medium should indicate the pH by changing from intense yellow to pink). In addition, use a pH strip.

3.1.3) Carefully add 2 mL of 1X DMEM and mix the solution. Plate approximately 2 mL of collagen in each well of the steel rectangular mold (20x30x10 mm) and incubate at 37^{o} C in 5% CO_2 for 10 min.

NOTE: The concentration of collagen should be 2.06 mg/mL in 0.6% acetic acid and the amount of collagen 1 ml/cm2.

3.1.4) Once the collagen sets into the mold, place the biomaterial (PLGA) on top of the collagen gel (20x30 mm) and pour the remaining collagen (about 6 mL) on top of it. Incubate at 37^{0} C in 5% CO_2 for 20 min.

3.1.5) Place the minced tissue (for 1:6 expansion) on the top of collagen gel. Press the water out of the construct by mechanical force using plastic compression as follows (Figures 3 and 4).

(An animated cartoon depicting Figure 4 could be added in the video)

3.1.6) Place a thick layer of gauze pads on a sterile surface. Place one stainless steel mesh (400 µm thick) on top of the gauze pads and then a sheet of nylon mesh (110 µm thick). Carefully transfer the collagen gel/minced tissue onto the nylon mesh and carefully remove the rectangular steel mold.

3.1.7) Place a new layer of nylon mesh on top of the collagen gel/minced tissue. Place a second steel mesh on top of the nylon mesh. Place in position the pressure or loading plate weighing a minimum of 120 g (i.e., a glass plate) for 5 min.

3.1.8) Remove the weight, nylon, and steel meshes. The autografts are now ready to be sutured to the pig bladder in the full-thickness skin wounds or cultured *in vitro.*

3.1.9) For *in vitro* culturing, cut the thin construct into small pieces fitting 12-well plates. Add 1 mL of keratinocyte medium. Place the plates in the incubator at 37^{o} C, 5% CO_2 and culture up to 6 weeks and change the medium 3 times per week.

4) Suture of autografts

4.1) Suture of autograft with minced bladder mucosa to the pig bladder

4.1.1) Keep the autograft moist in DMEM during the waiting time. Suture the autograft with fine running monofilament sutures. Use non-absorbable 5-0 Ethilon for research purposes.

4.1.2) Check if watertight by filling the bladder with saline through the indwelling urinary catheter. If possible, cover the autograft with a layer of greater omentum. Close the abdominal wall, subcutaneous tissue, and skin as described in 1.2.6. Apply a wound dressing.

4.2) Suture of the autograft with minced skin epidermis to a full-thickness wound

4.2.1) Keep the autograft moist in DMEM during the waiting time. Suture the autograft to the bottom of the skin full-thickness wound by interrupted sutures in the corners and in the middle of the autograft to keep the autograft closely attached to the underlying surface. Cover the wound with a plastic dressing that keeps the wound moist.

5) Termination

5.1.1) Sedate the animal with intramuscular injection of zolazepam hypochloride (2.5 mg/kg) and medetomidine (25 µg/kg) prior to termination and apply monitoring devices to the ear or tail to check for pulse and blood pressure.

5.1.2) Euthanize the animal by administering a lethal dose of pentobarbital sodium (60-140 mg/kg) intravenously. Check pulse and blood pressure until death has occurred.

6) *In vitro* culture

NOTE: To evaluate histologically the progression of the minced tissue in the PCL/collagen constructs *in vitro*, the collagen/PCL/minced patches are cultured in 12-well plates using keratinocyte medium.

6.1) Preparation of keratinocyte medium:

6.1.1) Sterilize a 500 mL glass bottle.

6.1.2) Mix 400 mL of DMEM with 100 mL of Ham's F12 (4:1 mixture). Supplement with 10% fetal bovine serum, 5 µg/mL insulin, 0.4 µg/mL hydrocortisone, 21 µg/mL adenine, 10^{-10} mol/L cholera toxin, $2x10^{-9}$ mol/L triiodothyronine, 5 µg/mL transferrin, 10 ng/mL epidermal growth factor, 50U/mL penicillin and 50 µg/mL streptomycin.

6.1.3) Sterilize by filtrating through a 0.2 µm filter and collect the filtrate in the sterile 500 mL bottle.

7) Immunohistochemistry

NOTE: The immunohistochemistry protocol is generally divided into the following steps: (1) fixation and paraffin embedding, (2) micro-sectioning to 5-µm slices, placement on slides, deparaffination, and rehydration, (3) antigen unmasking, staining and mounting. Before starting the last steps in the immunohistochemistry procedure, prepare the washing buffers and the antigen unmasking solution (see separate material details). Prepare the ABC complex solution at least 30 min before use.

7.1) Fixation

NOTE: At the end of the *in vitro* culture, fix the patches as follows:

7.1.1) Prepare Eppendorf tubes with 1 mL of 4% buffered formaldehyde (PFA) (Caution:

formaldehyde is toxic. Please read material safety data sheets before working with this chemical. Wear gloves and safety glasses and prepare the solution inside a fume hood).

7.1.2) Transfer each of the collagen patches to an Eppendorf tube containing 4% PFA. Fix over night at room temperature.

7.1.3) Place samples in 70% ethanol for long-term storage at 4°C. Samples are now ready for dehydration and embedding in paraffin blocks before sectioning.

7.2) Rehydration

7.2.1) Place the slides in a staining jar with X-tra solv for 15 min. Repeat by using a new staining jar with X-tra solv. Place the slides in a staining jar with absolute ethanol for 10 min. Repeat by using a new staining jar with absolute ethanol. Place the slides in a staining jar with 95% ethanol for 10 min and thereafter into a staining jar with 70% ethanol for 10 min. Finally wash the slides twice for 5 min with distilled water.

7.3) Antigen unmasking

7.3.1) Put slides in a Coplin jar with TE-solution and put the jar in a water bath to boil for 20 min. Take the jar out of the water bath carefully. Cool the slides to room temperature for 30 min and wash twice for 5 min in Tris buffer. Place the slides in a staining jar with 3% hydrogen peroxide for 10 min. Wash the slides twice for 5 min in Tris buffer. Draw a circle around the samples using a water repellent marking pen.

7.3.2) Block nonspecific binding of the antibody using 100–300 µL of blocking solution. Remove the blocking solution and add 100–300 µL of primary antibody dissolved at the recommended concentration in Tris buffer. Incubate overnight. Remove the antibody solution and wash sections in Tris buffer twice for 5 min.

7.3.3) Incubate with the secondary antibody for 1 h at room temperature. Wash twice for 5 min in Tris buffer. Incubate 30 min using the ABC Elite Kit (follow the manufacturer's instructions). Wash twice in Tris buffer.

7.3.4) Develop antibody reaction by using the Vector VIP Kit, following the manufacturer's instructions (1–7 min incubation generally produces a clear violet intensity). Put the slides in distilled water. Counterstain with Mayer's hematoxylin for 30 sec.

7.3.5) Wash in running water for 5 min. Place the slides in a staining jar with 70% ethanol for 1 min. Repeat by using a new staining jar with 70% ethanol. Place the slides in a staining jar with 95% ethanol for 1 min. Repeat by using a new staining jar with 95% ethanol.

7.3.6) Place the slides in a staining jar with X-tra solv for 5 min. Remove, one at a time to keep moist. Place a drop of mounting medium on top of each slide and put a cover glass on top (do so carefully to avoid air bubbles). Let the slides dry overnight and view slides under a microscope.

REPRESENTATIVE RESULTS:

This study presents a method that shows how to produce a biomaterial for transplantation

using plastic compression of collagen and minced tissue.

Epithelium and skin can be harvested and then mechanically minced into small particles (Figure 3). By plastic compression, the minced particles are incorporated within the composite scaffold composed of a centrally placed biodegradable polymer that is mechanically strong within outer layers of a collagen gel (Figure 4). Minced tissue particles can be separated to allow a 1:6 expansion rate. By pressing out the water content of the collagen, a biotransplant for autologous reconstructive surgery is completed.

Biotransplants can be used for autotransplantation to the animal for *in vivo* research studies or cultured in an ordinary cell culture environment for further *in vitro* studies.
The composite autograft allows grasping and suturing and sustains surgical handling (Figure 5). The process from tissue harvest and preparation of the cell-containing autograft for reconstructive surgery takes approximately 20 minutes and is aimed to be performed as a single-staged procedure.

In *in vitro* studies cells migrate, expand and reorganize to the surface of the autograft into a single-cell continuous layer in two weeks. After four weeks, the continuous epithelium is approximately 4 cell layers thick (Figure 6). Immunohistochemistry at different points in time reveals that cells proliferate, migrate, and reorganize into a continuous epithelium with a microarchitecture typical for the cell phenotype. The same results apply for autografts with minced skin epithelium as for minced bladder mucosa.

FIGURE LEGENDS:
Figure 1: Meek theory. According to Meek's theory, epithelium grows from the edges of a wound. By increasing the area exposed by the mincing technology, minced tissue epithelializes wounds from many spots.

Figure 2: Animated cartoon of an *in vivo* intracorporal tissue expansion according to the theory of Meek. By using minced tissue placed on a mold and then transplanted to the subcutaneous tissue, the hypothesis is that the epithelial cells migrate from the edges of the minced tissue, reorganize, and expand so as to form a continuous neoepithelium that covers the wound area and separates the foreign body (the mold) from the inner body.

Figure 3: Minced tissue preparation and plastic compression. The preparation of bladder mucosa (A) for mincing (C) and plastic compression (D–F), using the mincing device in (B) and the mold in (D) to produce a 420-μm-thick transplant (H). Note that collagen itself is a good extracellular matrix but is difficult to handle mechanically (G); by placing a biodegradable fabric as a reinforcing core, the transplant becomes easy to grasp (H).

Figure 4: Plastic compression
An animated cartoon showing the process of plastic compression with minced particles.

Figure 5: Surgical handling
Full-thickness skin wound model in a rat demonstrating sutured plastic-compressed biomaterial with minced skin for autotransplantation marked as T (transplanted autograft) Sutured biomaterial without minced skin marked as S (sham). In this case, minced skin

epithelium was transplanted for a 1:3 expansion rate.

Figure 6: Immunohistochemical staining to visualize the progression of the 3D culture in the collagen-biomaterial minced skin and bladder mucosa.
(A–D) Hematoxylin/eosin staining of (A) native skin, (B) native bladder, (C) minced skin after 5 weeks in culture and (D) minced bladder after 6 weeks in culture. The expression of epithelial and proliferation markers with MNF116 (E) and Ki 67 antibodies (F), respectively, after 2 weeks in culture.

DISCUSSION

This study presents an easy-to-use approach to produce bladder wall patches with autologous tissue for transplantation at the surgical table. The patches are formed by the combination of a biodegradable polymer knitting in the middle and collagen with and without minced tissue in the outer surfaces in combination with plastic compression. Plastic compression is a method previously described by other authors and can be defined as a rapid expulsion of fluid from collagen gels[12,13]. Minced tissue of bladder mucosa or skin is seeded into this scaffold and the formation of a skin or bladder epithelium could be followed during 6 weeks. Immunohistochemical analyses showed the formation of equivalents characteristic of normal tissue. These results allow not only an *in vitro* model for studying re-epithelialization and wound healing, but also forms a biomaterial for autologous tissue transplantation. Most importantly for urology purposes, the autografts may be used for *in vivo* applications of tissue engineering in reconstructive urology as engineered autologous tissue patches without the requirement for *in vitro* culture before transplantation[11].

In respect to cell expansion and *in vitro* culturing techniques, skin epithelium and uroepithelium share common characteristics. The motive to present the technique for plastic compression and mincing for both epithelial tissues in this study was that harvesting and *in vivo* studies for skin epithelium is easier than for bladder uroepithelium. By these means, biomaterials and skin epithelium can be studied as a first step to confirm biocompatibility and physical properties before performing more invasive studies in the urogenital organs.

In previous *in vivo* studies, using the concept of minced tissue with bladder mucosa for tissue regeneration of conduits, findings were that the small transplanted particles required some mechanical support in order to withstand mechanical trauma and to stay in place during the initial take of the transplanted minced particles and during the healing process and tissue regeneration[6,7].

In order to overcome these weaknesses, the study aimed at developing a hybrid construct with a biodegradable core of a polymer knitted fabric and plastic-compressed collagen[11,14]. The collagen provides a favorable surface for cell attachment and growth and allows direct and rapid integration of the minced tissue into the scaffold. However, since the mechanical properties of collagen, even after plastic compression, are still weak and would not sustain the contractions and extensions of the natural bladder movements, a supporting core scaffold for the collagen was added. For more complex structures, transplanted tissue could be expanded around a prefabricated mold to produce a three-dimensional epithelialized

structure with a central lumen.

In these studies we used PLGA as a stabilizing polymer and the core of the biograft. PLGA is widely used in a variety of biomedical devices such as grafts, sutures, implants and prosthesis and is therefore well characterized. It demonstrates strong biocompatibility and has been shown to promote blood vessel growth[14]. In order to procure maturation of the regenerated tissue, i.e. innervation, smooth muscle tissue and extra cellular matrix, we chose a PLGA polymer with a slow degradation rate over several months. However, the degradation rate can be regulated by choice of polymer, thickness and density[11,16].

Collagen was chosen as a component of the composite construct due to well-known biocompatibility, safety and good healing characteristics. The collagen promotes ingrowth of granulation tissue, re-modelling and maturation of extra-cellular matrix[15]. As collagen is degraded, neovascularization and granulation tissue supports the transplanted minced particles that reorganize, migrate and expand[5-7,11,16]. Besides, collagen is a major natural component of the extracellular matrix both in skin and in the bladder and has been used for creation of scaffolds both *in vitro* and *in vivo* including wound healing studies and reconstructions in the urogenital system[17,18].

The critical steps within the protocol are easily manageable. First, the biopsy specimens have to be kept under moist and favorable conditions prior to insertion into the transplant or else cell growth will be delayed or absent. A second pitfall may involve getting the right solidity of the collagen. One must make sure that the collagen becomes stiff during the preparation of the autograft by avoiding bubbles inside the collagen gel. Bubbles are avoided by careful pipetting and small bubbles can be ruptured with a needle. Collagen with bubbles should be discarded.

It is also important to get the right size of the minced tissue; before mincing the tissue, make sure that only the thin bladder mucosa is used in cases of bladder expansion or only the epidermis in cases of skin expansion. The last pitfall is when suturing: make sure to move perpendicularly to the transplant with the needle in order to avoid separating the different layers. Edges of the autograft also need to be handled carefully in order not to separate the collagen from the polymer. After suturing the sheets will be harder to separate. These are common problems that may arise when learning the technique.

One limitation of this technique is that the cells in the minced particles rely on diffusion of nutrients and oxygen. Therefore, the thickness of the autograft has to be less than 1 mm and has to be placed in a well-vascularized site. This might be a disadvantage due to risk of leakage of urine through the transplanted part of the bladder. Depending on the plastic compression, different permeability constants can be achieved. In our case the hydraulic permeability (k) value was 0.034 according to previous studies[19]. In a clinical setting we anticipate that we would need to keep the bladder empty with catheters for approximately 1-2 weeks in order to give time for the urothelial cells to build up a multi-layered continuous urothelium that make the patch un-permeable before active use.

Another limitation is the assumption of a healthy organ for tissue harvest and expansion. In the more severe cases when the bladder is missing or is unfit for expansion, for example,

when affected by cancer, the technique might not be suitable.

As for other tissue engineered cell constructs, final conclusions on functional and morphological results can only be answered in long-term *in vivo* studies. Our next step will be to analyze our results in long-term animal studies in respect to viability and physiological characteristics post-transplantation.

The significance of the presented technique is the possibility to expand tissue *in vivo* after only a single surgical procedure. Other techniques currently being evaluated are all dependent on expanding the cells *in vitro* before transplantation. The *in vitro* procedures have the downside of being associated with high costs, requiring advanced laboratory personnel, and being time- consuming. It may take up to 2 months between the harvesting of tissue or cells and a tissue-engineered autograft; as opposed to less than 1 hour, according to the technique described in this study.

In the future, minced tissue in compressed collagen techniques may be expanded and used in other organs, such as abdominal wall defects, diaphragmatic hernias, and other conditions where a patch? is not easily reconstructed with existing tissue.

In conclusion, an easy-to-perform method is described for transplantation of skin and bladder mucosa that can expand *in vivo*. The procedure of preparing an autograft is easy to do and can be used under sterile conditions in an ordinary surgical setting. The autograft resists surgical handling and is biodegradable. The core of the autograft can be composed of different biodegradable polymers depending on preferences regarding the rate of degradation and other characteristics, such as elasticity, thickness, and porosity, and according to the patient's specific needs. In a clinical setting, the patient would undergo tissue harvest, preparation of the composite autograft and autotransplantation as a single-stage procedure. In addition, all parts of the cell-containing bioconstructs are currently FDA approved.

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DISCLOSURES

The authors have nothing to disclose.

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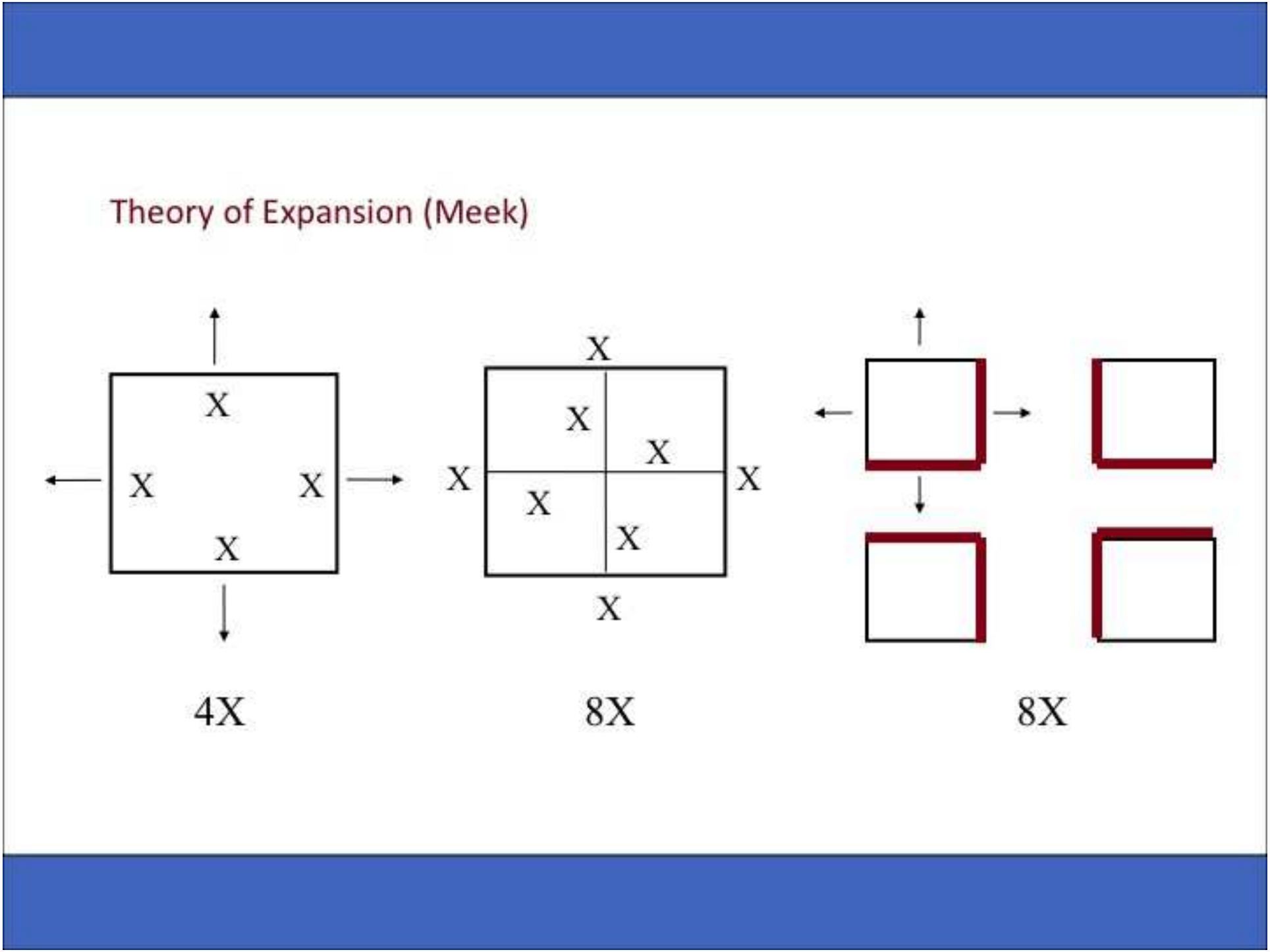

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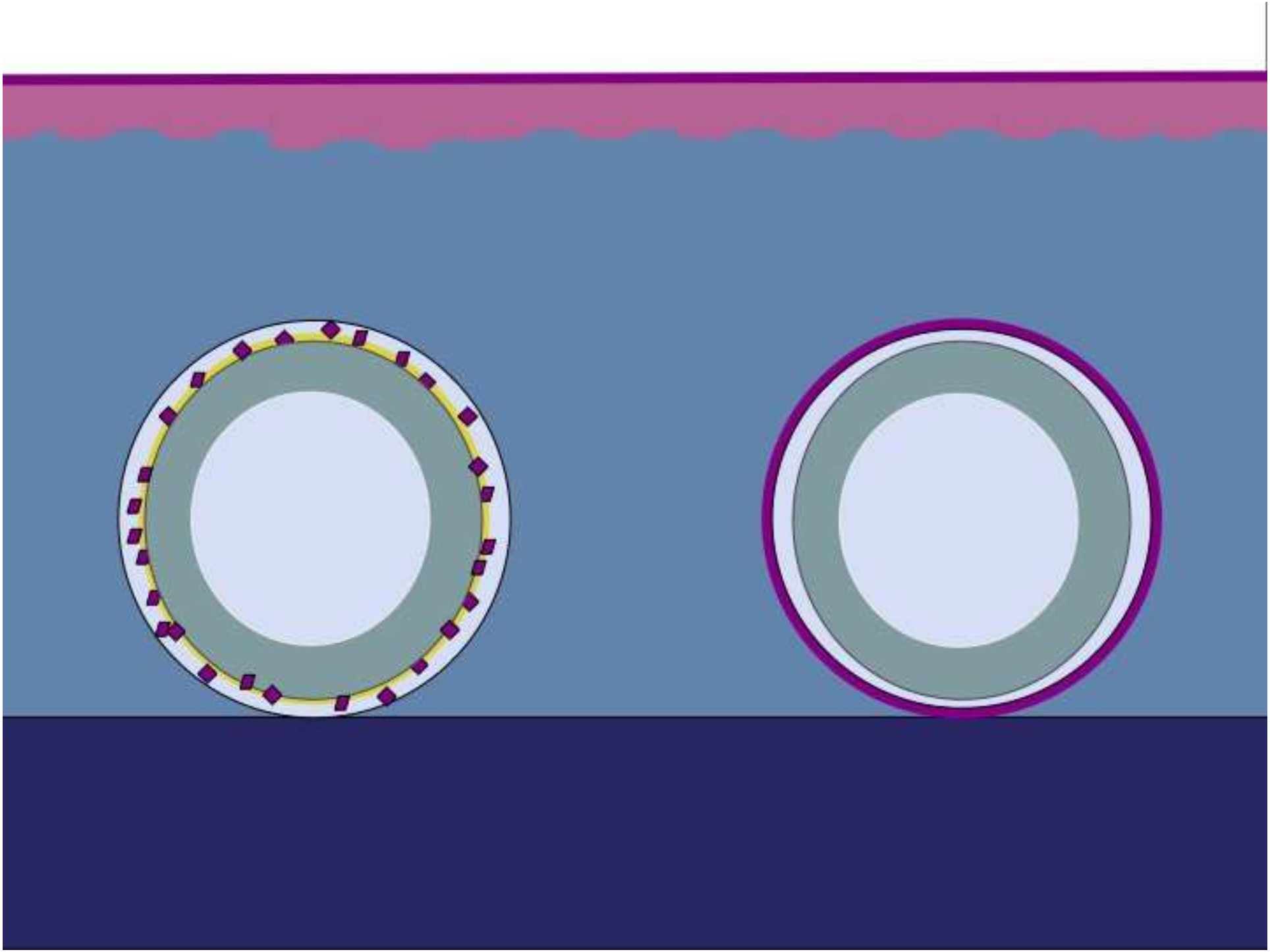

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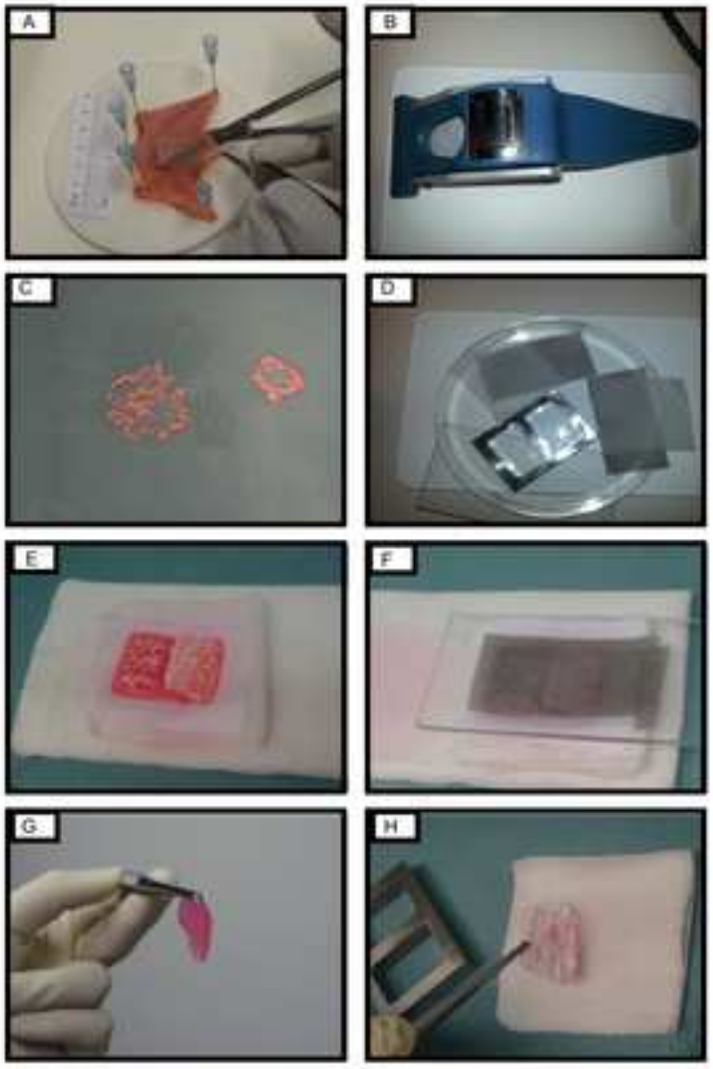

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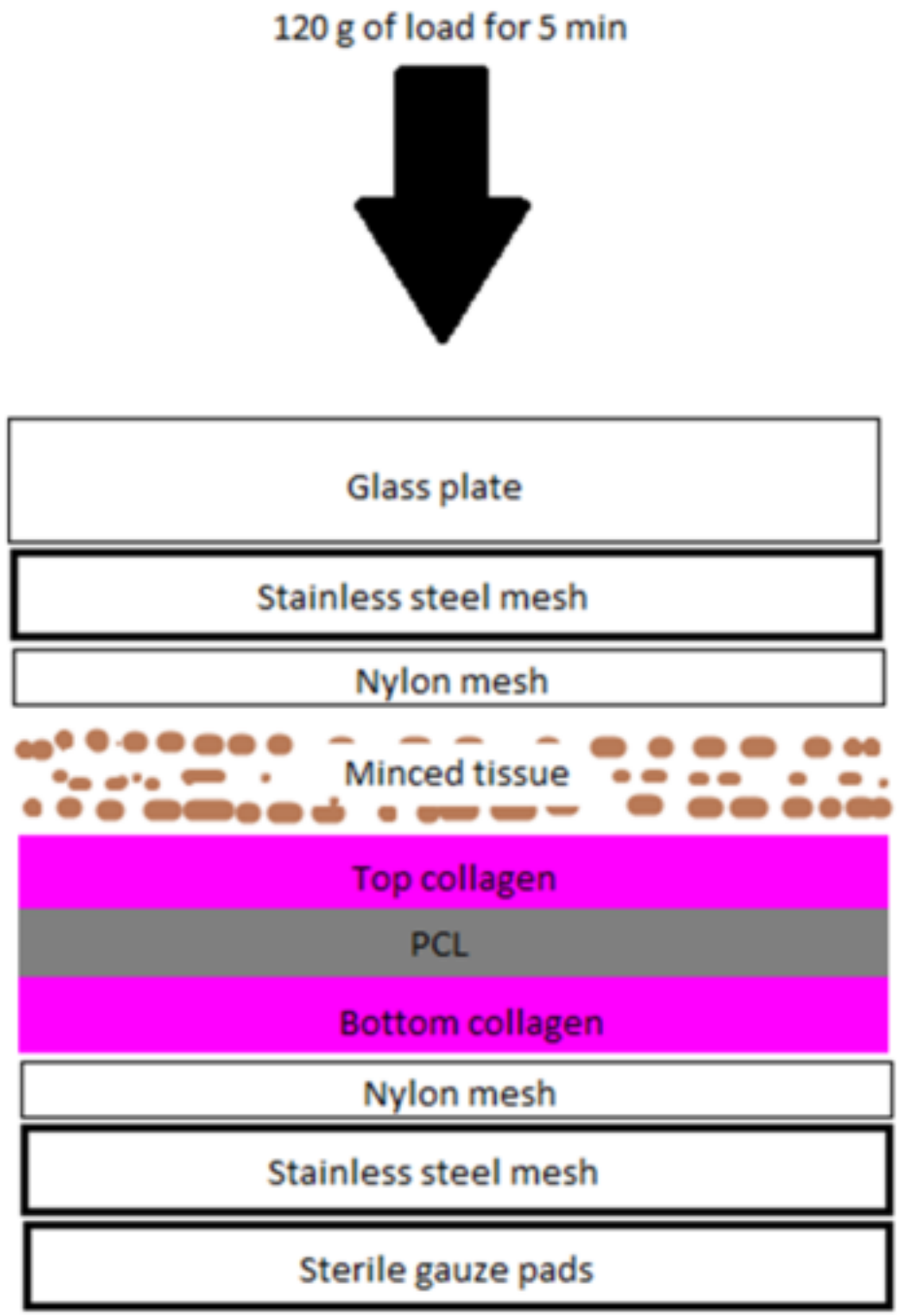

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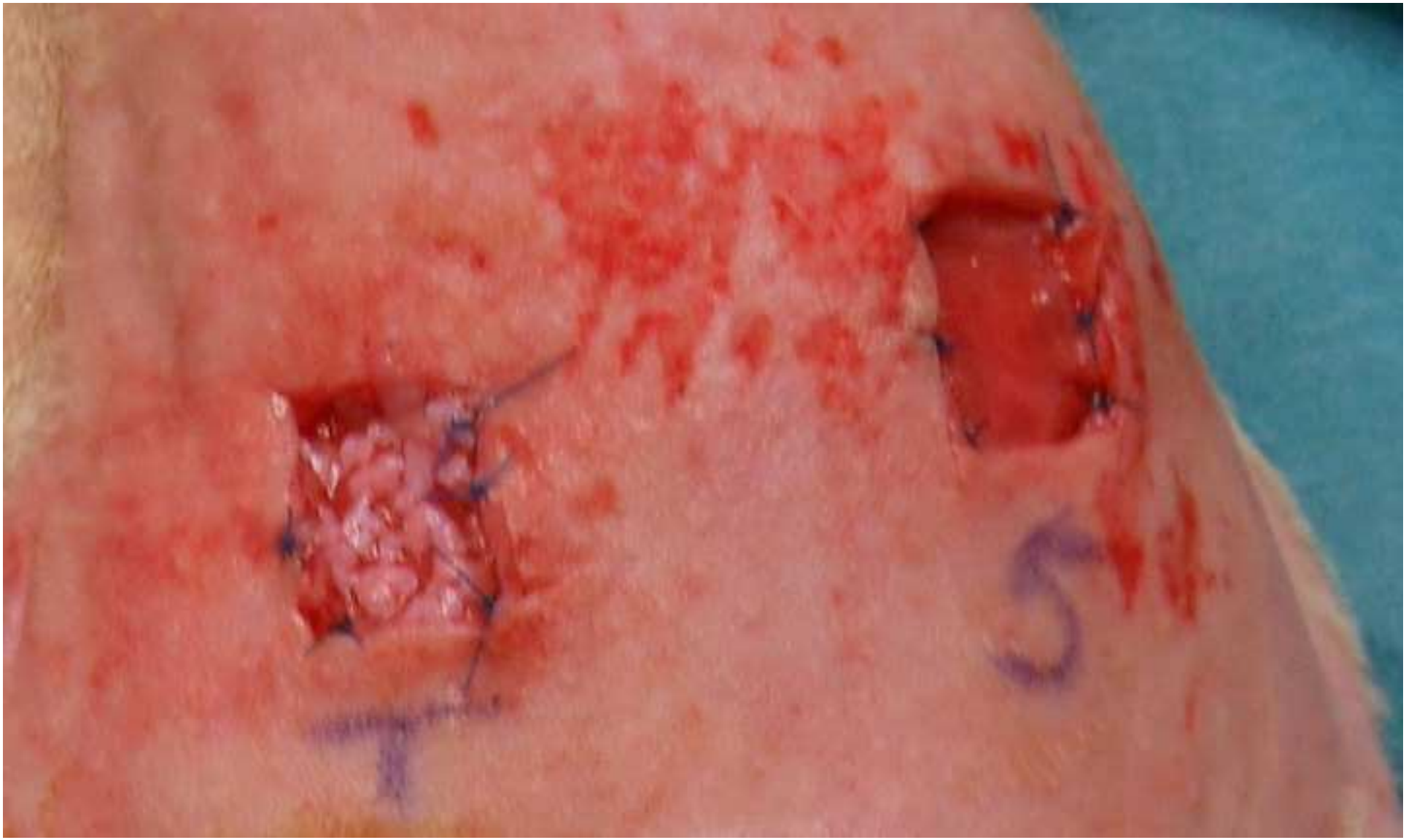

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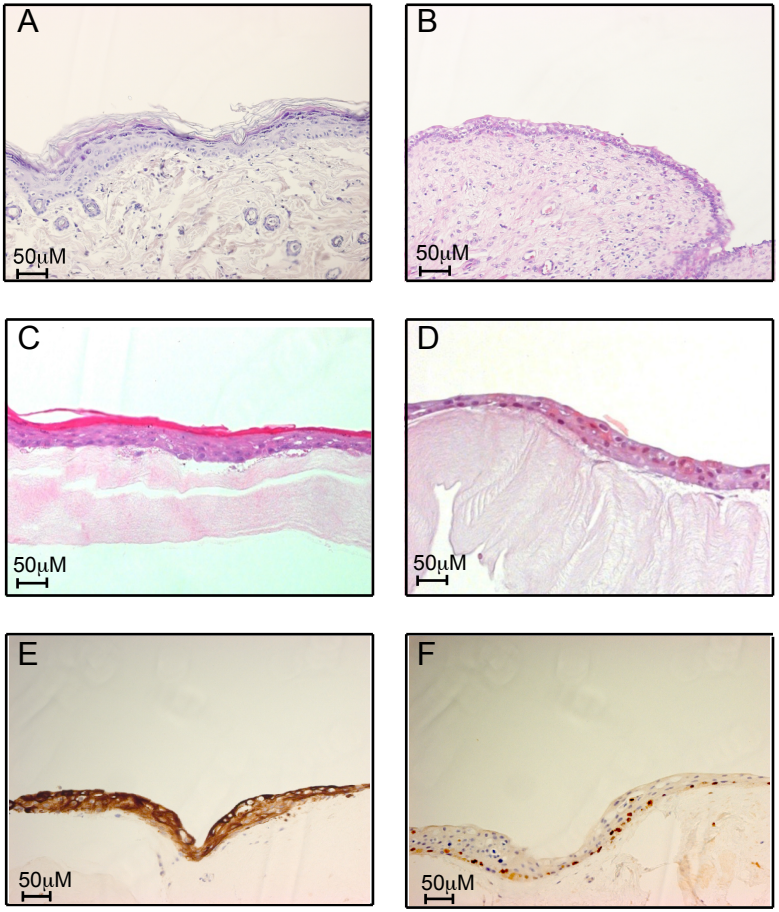

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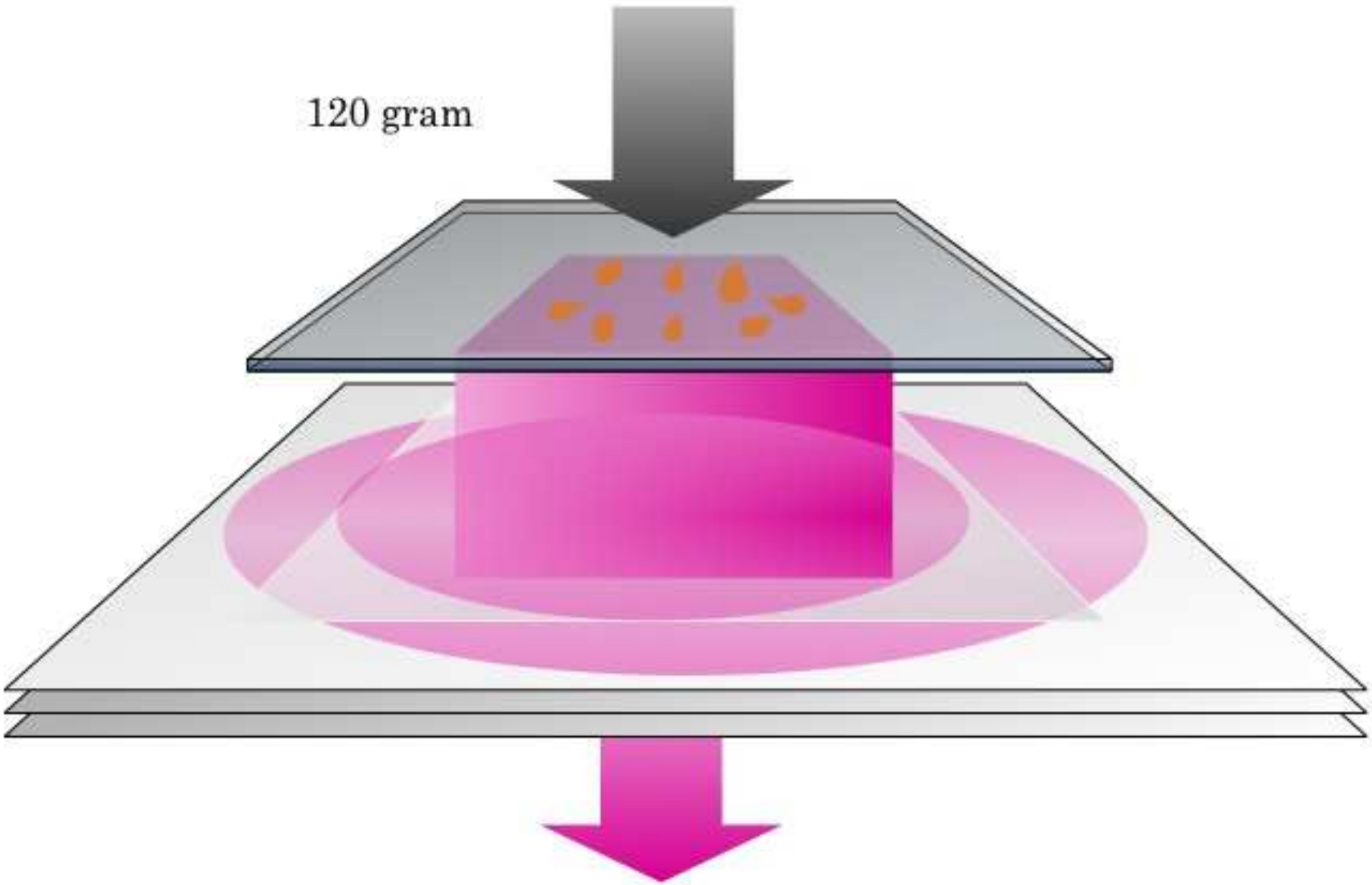

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Name of Reagent/ Equipment
Silicone catheter 10-French
DMEM 10X
24 well plates
3'3,'5-Triiodothyronine
4% PFA
70% ethanol
ABC Elite kit: Biotin -Streptavidin detection kit
Absolute ethanol
Adenine
Atropine 25 µg/kg
Azaperone 2 mg/kg
Biosafety Level 2 hood
Blocking solution: Normal serum from the same species as the secondary secondary antibody was gener
Buprenorphine 45 µg/kg
Carprofen 3 mg/kg
Chlorhexidine gluconate
Cholera toxin
Coplin jar: staining jar for boiling
Stainless mold (33x22x10 mm) custom made
DMEM
Epidermal growth factor
Ethilon (non-absorbable monofilament for skin sutures)
Fetal bovine serum (FBS)
Forceps (Adison with tooth)
Gauze (Gazin Mullkompresse)
Ham´s F12
Hematoxylin
Humidity chamber
Hydrocortisone
Hydrogen peroxide Solution 30%
Insulin
Isoflurane
Lidocaine 5 mg/ml
Lucose 25 mg/mL
Marker pen pap pen
Medetomidine 25 µg/kg
Mincing device
Monocryl (absorbable monofilament)
NaCl
NaOH 1N
Nylon mesh, 110 uM thick pore size 0.04 sqmm
Oculentum simplex APL: ointment for eye protection
PBS

Penicillin-Streptomycin
Phenobarbiturate 15 mg/kg
PLGA Knitted fabric
Rat-tail collagen
Scalpel blade - 15
Shaving shears
Stainless stell mesh, 400 uM thick pore size
Steril gloves
Sterile gowns
Sterile drapes
Sterilium
Suture Thread Ethilon
TE-solution (antigen unmasking solution) consist of 10 mM Tris and 1 mM EDTA, pH 9.0
Tiletamine hypochloride 2,5 mg/kg
Transferrin
Trizma Base, H_2NC
Vector VIP kit: Enzyme peroxidase substrate kit
Vicryl (absorbable braded)
Tris buffer pH 7.6 (washing buffer)
X-tra solv (solvent)
Zolazepam hypochloride
Depilatory wax strips
Pentobarbital sodium

Company	Catalog Number
Gibco	31885-023
Falcon	08-772-1
Sigma-Aldrich	IRMM469
Labmed Solutions	200-001-8
Histolab	
Vector	PK6102
Histolab	1399.01
Sigma-Aldrich	A8626
Britain	
Stresnil, Janssen-Cilag, Pharma, Austria	
Vector	The blocking solution depends of
Atropin, Mylan Inc, Canonsburg, PA	
Rimadyl, Orion Pharma, Sweden	
Hibiscrub 40 mg/mL, Regent Medical, England	
Sigma-Aldrich	C8052
Histolab	6150
Gibco	3188-5023
Sigma-Aldrich	E9644
Ethicon	
Gibco	10437-036
Gibco	31765-027
Histolab	1820
DALAB	
Sigma-Aldrich	H0888
Sigma-Aldrich	H1009
Sigma-Aldrich	I3536
Isoflurane, Baxter, Deerfield, IL	
Xylocaine, AstraZeneca, Sweden	
Baxter, Deerfield, IL	
Sigma-Aldrich	Z377821-1EA
Domitor, Orion Pharma, Sweden	
Applied Tissue Technologies LLC	
Ethicon	
Sigma-Aldrich	S7653
Merck Millipore	106462
APL	Vnr 336164
Gibco	14190-094

Gibco	15140-122
Pentobarbital, APL, Sweden	
First LINK, Ltd, UK	60-30-810
Bode Chemie HAMBURG	
Sigma-Aldrich	T8158
Sigma-Aldrich	T6066
Vector	SK4600
Ethicon	
DALAB	41-5213-810
Zoletil, Virbac, France	
Veet	
Lundbeck	

Comments/Description
Preparing the animal for surgery , Section 1
Plastic compression section 4
Plastic compression section 4
In vitro culture; Section 5
Immunocytochemistry; Section 6
Immunocytochemistry; Section 6
Immunocytochemistry; Section 6
Immunocytochemistry; Section 6
In vitro culture; Section 5
Preparing the animal for surgery , Section 1
Preparing the animal for surgery , Section 1
Plastic compression; Section 4
Immunocytochemistry; Section 6
Preparing the animal for surgery , Section 1
Preparing the animal for surgery , Section 1
Preparing the animal for surgery , Section 1
In vitro culture; Section 5
Immunocytochemistry; Section 6
Plastic compression; Section 4
Plastic compression section 4. Keep on ice when using it in plastic compression
In vitro culture; Section 5
Surgery, Section 1
Plastic compression section 4
Preparing the animal for surgery , Section 1
Preparing the animal for surgery , Section 1
Plastic compression section 4
Immunocytochemistry; Section 6
Immunocytochemistry; Section 6
In vitro culture; Section 5
Immunocytochemistry; Section 6
In vitro culture; Section 5
Preparing the animal for surgery , Section 1
Preparing the animal for surgery , Section 1
Preparing the animal for surgery , Section 1
Immunocytochemistry; Section 6
Preparing the animal for surgery , Section 1
Minced tissue preparation, section 2
Surgery, Section 1
Immunocytochemistry; Section 6
Plastic compression section 4 and cell culture
Plastic compression; Section 4
Surgery, Section 1
Plastic compression section 4

Plastic compression section 4
Preparing the animal for surgery , Section 1
Plastic compression; Section 4
Plastic compression section 4, keep on ice
Preparing the animal for surgery , Section 1
Preparing the animal for surgery , Section 1
Plastic compression; Section 4
Preparing the animal for surgery , Section 1
Preparing the animal for surgery , Section 1
Preparing the animal for surgery , Section 1
Preparing the animal for surgery , Section 1
10 mM Tris/1 mM EDTA, adjust pH to 9.0
Preparing the animal for surgery , Section 1
In vitro culture; Section 5
Immunocytochemistry; Section 6
Immunocytochemistry; Section 6
Surgery, Section 1
TE solution: Make 10X (0,5M Tris, 1,5M NaCl) by mixing: 60,6 g Tris (Trizma Base, H2NC(CH2OH)3, M=121.14 g/mol), add 800 ml distilled water adjust the pH till 7.6, add 87,7 g NaCl and fill to 1000 ml with distilled water. Dilute to 1X with distilled water.
Immunocytochemistry; Section 6. Use under fume hood
Preparing the animal for surgery , Section 1
Preparing the animal for surgery , Section 1
Termination, Section 3

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Magdalena Fossum, MD PhD

27 March 2015

Answers to the editorial comments regarding manuscript JoVE53061R2
"Minced tissue in compressed collagen- a cell containing bio-transplant for single-staged reconstructive repairs"

The authors would like to thank the editors and reviewers for the comments given and hope you find our improvements suitable for accepting the manuscript for publication in JoVE. Changes in the manuscript have been high-lighted in grey.

Editorial comments:
1. 4.1.5 - All action items need to be in the imperative tense.
Author response: All action items have been rewritten in imperative tense in the protocol.

2. Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.
Author response: Previous editorial comments have been taken in account when re-writing the manuscript.

3. Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.
Author response: Proofreading has been made.

4. If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.
Author response: No figures or tables have been published previously.

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from PubMed. In these cases, please manually include DOIs in reference information.
Author response: DOIs have been included in all references besides ref 4 (web page) and ref 9 (DOI not found). For ref 9 instead we added the Accession No. from Ovid.

Reviewers' comments:

Reviewer #2:
The data presented in vitro shows epithelial development which is good. But in vivo data is a significant lack - indicating that this is the authors next step would be good.
Editors Note:We do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.
Author response: An additional paragraph has been added under Discussion that further emphasizes the limits of the study and future steps needed to evaluate long-term *in vivo* results. (Page 13, 3rd paragraph).

As an alternative, the authors suggest that only the pre-operative *in vitro* part of the technique is presented in this publication. By these means only the "making of the autograft" and the histology after *in vitro* culturing would be described.

What is missing is a description of how the material would cope immediately post implantation where inflammation and re-modelling will severely degrade the collagen portion of the implant. The survival of the implant would be prey to a a failure to vascularise and integrate - the fate of the scaffold material would be the basis if its own invivo study to show whether its degradation causes delamination or if encapsualtion ould lead to failure.
Author response: Collagen has been used for surgical reconstructive surgery for long time and is known to degrade as new granulation tissue is being formed (ref 15). This is also what we experience from our on-going *in vivo* studies. A new reference has been added in addition to a paragraph in Discussions. (Page 12, 3rd paragraph, ref 15)

Additionally, the tissues being investigated are required to be water impermeable barriers and some indication of whether this will be the case from insertion. Some indication of the differing need of bladder and skin would be useful -

Author response: Water impermeability is not expected before a confluent multilayered urothelium has been developed. The autograft has not been constructed to be water impermeable *per se* but rather it should permit diffusion of water and nutrition.

During the time for take and healing, the bladder will be off-loaded with an indwelling catheter. This has further elaborated in a new paragraph in Discussions. (Page 11 and 12, last and 1st paragraph, ref 19)

Regarding the motive to study both bladder and skin in this context has been further discussed in Discussions. (Page 1, 2nd paragraph in Discussions)

Minor Concerns:

A minor quibble is the use of 'transplant' - suggesting donor tissue. I think autograft better describes the material being produced.

Author response: The authors agree that the use of autograft is appropriate in this context and changes have been made accordingly throughout the manuscript. (Changes highlighted in grey throughout the manuscript)

Reviewer #3:

Major Concerns:

In order to have the approach standardized and become applicable as routine approved protocol, some basic approaches should be taken. Answers to the following question might help to address the fundamental characterization means and criteria needed to have the article qualified for publication.

* What are the physical properties (density, mesh size, fiber thickness, mechanical strength, wet-ability and elasticity) of the electrospun mat of PLGA scaffold?

Author response: The physical properties of the polymer inside the collagen sheets can be regulated. Depending on which polymer you use you can change its properties. Our polymer of choice has been a PLGA, but we have also used PCL. The properties of these polymers have been investigated in previous papers (ref. 11,16). This matter has been further elucidated in the text (page 12, 2nd paragraph).

* What is the actual amount, concentration and/or ratio of collagen with respect the desired area of formed mold?

Author response: 1ml/cm2 collagen before plastic compression. Concentration of collagen before plastic compression is 2.06 mg/mL protein in 0.6% acetic acid. This information has been added accordingly in the protocol (Page 7, paragraph 3.1.3).

How was the interlink of collagen sheets by means of PLGA characterized? How much is the risk of having the collagen sheets separated from each other leaving the cells accumulated between two permeable and non-stable sheets?

Author response: The interlink is described in references 11 and 16 for PCL and PLGA, respectively. You have to handle the edges of the autograft carefully not to separate the collagen from the polymer during surgery, but after suturing to the bladder wall is complete the sheets will be harder to separate. Further details regarding the suturing technique has been added (page 12, paragraph 5).

How strong the sheets are held together?
Author response: The construct allows mechanical handling and careful surgery. In our *in vitro* studies and preliminary *in vivo* studies, were the transplant is sutured subcutaneously, we have not seen delamination between the sheets.

How long does it take for the PLGA in the sandwich to be degraded? Is it long enough to have cells interwoven the sheets together?
Author response: The degradation of PLGA will be over several months, to give the cells more than enough time. In this context we want a slow degradation in order to procure regeneration and maturation of innervation, new smooth muscle tissue and extra cellular matrix including collagens in the bladder wall. Even here the degradation of the polymer can be regulated depending on which polymer you use and with what density and thickness. Further details have been added accordingly (page 12, 2nd paragraph).

What are the values (v/v, w/w, w (g)/area (cm2), ...) of the ratio of minced tissue to the collagen gel?
Author response: The ratio used for expansion has been 1:6, i.e. 1 cm2 of mucosa or epidermis for 6 cm2 of scaffold. Please see paragraph 3.1.5, page 7.

How much is the water permeability of the patches ready to be sutured into the pig bladder?
Author response: The collagen and polymer will need to be permeable for nutrients and waste to pass to and from the cells. Depending on the plastic compression, different permeability constants will be achieved. In our case a permeability constant of 0.034 is expected according to previous studies (Serposhan et al, 2010). In a clinical setting we would need to keep the bladder empty with catheters for approximately 1-2 weeks, to give the urothelium time to build a layer of epithelium and make the patch un-permeable before starting to use the bladder. (pages 12 and 13, last and first paragraph).

How are the mechanical strength, elasticity and stretchability of the graft? What were the means of characterisation?
Author response: Please see previous studies for tensile strength (ref 11 and 16). Elasticity for PLGA was 81 % elongation at break, but this can of course be regulated

depending on which polymer you use.

* Although the cells look to have been arranged in the grafts of skin and bladder models similar to the native ones, to some extent, morphologically, how comparable they are in terms of their function as skin and urothelium ?
Author response: As for other tissue engineered cell constructs, these questions can only be answered after long-term *in vivo* studies. We are currently performing *in vivo* studies and data collection and interpretations are ongoing. However, the answers to these questions are outside the range of the present study protocol. (Page 13, paragraph 3)

Minor Concerns:
* The individual sheets and constituents shown in the Figure 4 should be marked. Possible means to address the angiogenisis, innervation and changes in the matrix may be elaborated.
Author response: Figure 4 has been up-dated accordingly. The cartoon aims to describe the method of construction and preparation of the composite autograft for transplantation. *In vivo* changes, such as tissue regeneration and matrix degradation is therefore not included.
However, if possible we would like to include an animated cartoon that also demonstrates the anticipated angiogenesis, innervation and matrix degradation over time.

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28 January 2015

Answers to the editorial comments regarding manuscript JoVE53061R1
"Minced tissue in compressed collagen- a cell containing bio-transplant for single-staged reconstructive repairs**":**

1. Thank you so much for submitting your revised manuscript. All of your previous revisions have been incorporated into the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes (File name: 53061).
Answer: The file 53061 has been down loaded and revised.

2. Your current long abstract is over our 300 word limit; please modify your long abstract in both the manuscript document and your editorial manager account.
Answer: The abstract has been modified.

3. Please adjust the numbering of your protocol section to follow JoVE instructions for authors, 1. should be followed by 1.1) and then 1.1.1) if necessary and all steps should be lined up at the left margin with no indentations. For instance, step 1, fast the animal should be step number 1.1.1, Give Phenobarbiturate should be step 1.1.2. and so on.
Answer: The numbering has been corrected.

4. Additionally the steps in section 1.2 are not in order, see step 12, 16, 16 and "If biopsy…" statement is not numbered.
Answer: Corrections have been made accordingly.

5. Please check grammar throughout the manuscript. There are grammatical errors such as "Administrate an intramuscular… , "If the biopsy should not be used for autotransplantation…", etc. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues, preferably by a native English speaker. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Answer: Professional linguistic revisions have been performed.

6. Please re-write steps of your protocol section in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.). For instance, step 1 should be "Administer intramuscular injection…", Please check for such instances thoroughly in the protocol section.

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Answer: Revisions to imperative tense have been undertaken.

7. Please specify the dose of pentothal and fentanyl administered and the route.
Answer: Complementary information has been added.

8. Please revise the text to avoid the use of any pronouns (i.e. "we", "you", "your", “our” etc.). If you feel it is very important to give a personal example, you may use the royal "we" sparingly and only as a "NOTE:" after the relevant protocol step. Please use the Ctrl+F function to find and replace the pronouns.
Answer: Pronouns have been omitted throughout the text.

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Answer: Relevant steps are highlighted in yellow in the text.

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Answer: The reference list has been updated accordingly.

Sincerely yours,
Magdalena Fossum
Corresponding author