

## Preliminary data for the development of a decellularized membrane from animal dermis

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**Abstract:** Circumcision, the surgical removal of the foreskin, is a widespread practice that has been adopted in a variety of circumstances. Although circumcision was once justified on clinical grounds, evidence now demonstrates that circumcision is rarely indicated for only a handful of conditions. Decades of indiscriminate infant circumcision have left millions of men without a foreskin and open to the possibility of regenerative interventions. In this study we present preliminary data for the development of an *in vitro* decellularized membrane from animal dermis (ADM) using bull foreskin. A reduction of cell viability was identified in ADM tissue using the MTT cell viability test while histological analyses with Hematoxylin and Eosin (H&E) were performed to evaluate the maintenance of the ADM architecture.

**Objectives:** To develop a decellularized membrane from animal dermis derived from bull foreskin as a starting point to provide a solution to circumcised men by regenerative medicine approaches.

**Results:** All decellularization methods tested in our study were able to create ADM displaying a drastic reduction of cell viability whilst maintaining a normal tissue morphology and structure.

### Introduction

Circumcision, the removal of the foreskin, is a common surgical procedure. While most circumcisions are performed for religious and cultural reasons (Taylor et al., 1996; Senkul et al., 2003), circumcision is often a recommended treatment for clinical conditions such as redundant foreskin, phimosis, or lichen sclerosus (balanitis xerotica obliterans) of male genitalia. Some, despite conflicting evidence, recommend the procedure to prevent sexually transmitted infections and infectious conditions (Gerharz and Haarmann, 2000). Although attention to surgical technique can reduce the risk of postoperative complications, such as foreskin edema, substantial bleeding, pain, and infection (Wu et al., 2013; Arslan et al., 2013), these complications cannot be eliminated.

Several studies underline that foreskin removal interferes with normal human sexual functions. Histological analysis carried out by Taylor and coworkers (1996) identified the foreskin as a

highly innervated and vascularized erogenous tissue that plays a key role in normal sexual behavior (Taylor et al., 1996). Moreover, it was also identified that prepuce excision reduces both penile protection, and, in turn, its sensitivity (Sorrells et al., 2007; Bronselaer et al., 2013). Circumcision also alters the mechanical functioning of the penis, requiring a ten-fold increase in force to achieve vaginal penetration (Taves, 2002). Circumcision has also been associated with erectile dysfunction and difficulty in ejaculation (Dias et al., 2013). The physical and psychological problems that derive from prepuce amputation in circumcised men inspired Foregen onlus to seek a solution via regenerative medicine. In the present study, we provide preliminary data for the development of an *in vitro* decellularization method for animal derived dermis obtained from bull foreskin. The purpose is to create an animal derived membrane (ADM) for its possible future application on human foreskin tissue by Foregen onlus in an effort to treat the circumcised male using regenerative and reparative medicine.

## **Materials and Methods**

### **1. ADM procurement and decellularization**

Animal derived dermis was taken from foreskins of eight different bulls from a certified slaughterhouse. After obtaining the samples, the fresh tissues were maintained in physiological solution (NaCl 0.9% -Fresenius Kabi, Bad Homburg, Germany) (**Fig. 1A**) and then were processed with four different protocols of decellularization using biological agents (**Fig. 1B, 1C, 1D**) (Trypsin 2.5% 10x- Gibco® Life Technologies, NY, USA) in the BL3 Laboratory of Medical and Veterinary Science Department as described below:

- fresh tissues 1 and 5 were processed with decellularization protocol 1
- fresh tissues 2 and 6 were processed with decellularization protocol 2
- fresh tissues 3 and 7 were processed with decellularization protocol 3
- fresh tissues 4 and 8 were processed with decellularization protocol 4

#### **Protocol n.1**

Pre-freezing at -80 °C

Trypsin 1x for 48 h

Freezing at -80 °C

#### **Protocol n.2**

Trypsin 2x for 24 h

Freezing at -80 °C

#### **Protocol n.3**

Trypsin 3x for 24 h

Freezing at -80 °C

#### **Protocol n.4**

Trypsin 4x for 24 h

Freezing at -80 °C

Fresh tissues were also used as positive controls for biological characterization. All tissues were incubated at 37 °C in 5% CO<sub>2</sub>.

## **2. ADM assessment of decellularization**

The biological characterization of samples decellularized with the four different protocols described above was performed as follows:

- MTT cell viability test
- Histological analyses with Hematoxylin and Eosin (H&E)

To standardize the tissue-specific biological properties and to evaluate the intrinsic variability of tissue samples derived from different bulls, all fresh tissues were also characterized.

### **2.1 Cell viability test (MTT)**

Using a 6-mm biopsy punch, six uniform samples were taken from each fresh, decellularized dermal tissue. Two of them were soaked in liquid nitrogen for 10 minutes and used as negative controls. Tissue specimens were weighed, placed in a 12-well plate, and then incubated with 100 µl MTT (Roche Diagnostic GmbH, Penzberg, Germany) solution (0.5 mg/ml) for 3 h at 37 °C in an atmosphere of 5% CO<sub>2</sub>/air (**Fig. 2A, 2B**). Each tissue punch was then placed in 1 ml dimethyl sulfoxide (DMSO Bioniche Pharma USA LLC, Lake Forest, IL) for 10 minutes. The solution was read on a spectrophotometer at 570 nm, and DMSO was assayed as the background (**Fig. 2C, 2D**). For each sample, the viability rate was calculated as the ratio between the optical density (OD) at 570 nm and the weight in grams (gr). The viability index I<sub>v</sub> (+) was defined as the mean of the viability index I<sub>v</sub> (-) of the two negative controls.

### **2.2 Histological analysis with Hematoxylin and Eosin (H&E)**

Fresh ADM samples were fixed with 10% formalin solution and paraffin embedded. After processing, histological sections (5 µm in thickness) were stained with H&E for morphological analyses. Tissue architecture preservation was assessed qualitatively, as was the integrity of collagen and elastic fibers of cryo-preserved derma, in comparison with the relatively fresh sample. The specimens were examined with light microscopy, and the efficacy of the cell removal technique and the maintenance of tissue characteristics were verified.

## **3. Freezing and Storage**

All ADM were sealed inside cryofreezing bags in a solution containing RPMI 1640 medium plus L-Glutamine and Hepes 25mM (Pbi International), cryofrozen and stored at -80 °C (**Fig. 4**).

Only in the ADM 1 and 5 was a cryofreezing performed previously to tissue decellularization as described in protocol 1.

## Results

### Cell viability test (MTT) and histological analysis

Analysis of cell viability on eight fresh tissues used as positive controls and ADM were performed using the MTT test as described in Material and Methods (**Fig. 2, 3**). **Table 1** reports the optical density (OD) at 570 nm and the weight in grams of six uniform samples for each fresh tissue or ADM. Two of them were used as negative controls.

**Table 1**

| Fresh Tissue 1                       | Fresh Tissue 2                       | Fresh Tissue 3                     | Fresh Tissue 4                     | Fresh Tissue 5                       | Fresh Tissue 6                       | Fresh Tissue 7                       | Fresh Tissue 8                     |
|--------------------------------------|--------------------------------------|------------------------------------|------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|------------------------------------|
| 1(+)<br>0.0379 gr<br>0.890 OD        | 1(+)<br>0.03573 gr<br>0.796 OD       | 1(+)<br>0.0349 gr<br>0.913 OD      | 1(+)<br>0.0383 gr<br>0.986 OD      | 1(+)<br>0.0375 gr<br>0.860 OD        | 1(+)<br>0.0379 gr<br>0.890 OD        | 1(+)<br>0.0395 gr<br>0.964 OD        | 1(+)<br>0.0267 gr<br>0.890 OD      |
| 2(+)<br>0.0382 gr<br>0.950 OD        | 2(+)<br>0.0355 gr<br>0.832 OD        | 2(+)<br>0.0355 gr<br>0.894 OD      | 2(+)<br>0.0343 gr<br>0.909 OD      | 2(+)<br>0.0382 gr<br>0.895 OD        | 2(+)<br>0.0387 gr<br>0.854 OD        | 2(+)<br>0.0379 gr<br>0.854 OD        | 2(+)<br>0.0277 gr<br>0.992 OD      |
| 3(+)<br>0.0356 gr<br>0.840 OD        | 3(+)<br>0.0360 gr<br>0.872 OD        | 3(+)<br>0.0371 gr<br>0.919 OD      | 3(+)<br>0.0364 gr<br>0.946 OD      | 3(+)<br>0.0395 gr<br>0.993 OD        | 3(+)<br>0.0385 gr<br>0.882 OD        | 3(+)<br>0.0383 gr<br>0.952 OD        | 3(+)<br>0.0282 gr<br>0.957 OD      |
| 4(+)<br>0.0348 gr<br>0.790 OD        | 4(+)<br>0.0291 gr<br>0.873 OD        | 4(+)<br>0.0389 gr<br>0.950 OD      | 4(+)<br>0.0330 gr<br>0.899 OD      | 4(+)<br>0.0362 gr<br>0.745 OD        | 4(+)<br>0.0393 gr<br>0.958 OD        | 4(+)<br>0.0397 gr<br>0.874 OD        | 4(+)<br>0.0282 gr<br>0.936 OD      |
| 5(-)<br>0.03740 gr<br>0.650 OD       | 5(-)<br>0.0361 gr<br>0.751 OD        | 5(-)<br>0.0366 gr<br>0.911 OD      | 5(-)<br>0.0313 gr<br>0.870 OD      | 5(-)<br>0.0363 gr<br>0.530 OD        | 5(-)<br>0.0352 gr<br>0.724 OD        | 5(-)<br>0.0373 gr<br>0.753 OD        | 5(-)<br>0.0276 gr<br>0.759 OD      |
| 6(-)<br>0.0365 gr<br>0.590 OD        | 6(-)<br>0.0361 gr<br>0.721 OD        | 6(-)<br>0.0375 gr<br>0.973 OD      | 6(-)<br>0.0365 gr<br>0.869 OD      | 6(-)<br>0.0364 gr<br>0.560 OD        | 6(-)<br>0.0347 gr<br>0.642 OD        | 6(-)<br>0.0383 gr<br>0.788 OD        | 6(-)<br>0.0275 gr<br>0.743 OD      |
| $I_{V(+)}=94.63$<br>$I_{V(-)}=33.53$ | $I_{V(+)}=99.92$<br>$I_{V(-)}=40.77$ | $I_{V(+)}=100$<br>$I_{V(-)}=50.83$ | $I_{V(+)}=105$<br>$I_{V(-)}=51.59$ | $I_{V(+)}=93.33$<br>$I_{V(-)}=29.98$ | $I_{V(+)}=94.79$<br>$I_{V(-)}=39.06$ | $I_{V(+)}=93.49$<br>$I_{V(-)}=40.75$ | $I_{V(+)}=136$<br>$I_{V(-)}=54.51$ |

| ADM 1                                | ADM 2                              | ADM 3                               | ADM 4                                | ADM 5                               | ADM 6                                | ADM 7                                | ADM 8                                |
|--------------------------------------|------------------------------------|-------------------------------------|--------------------------------------|-------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
| 1(+)<br>0.0337 gr<br>0.215 OD        | 1(+)<br>0.0396 gr<br>0.474 OD      | 1(+)<br>0.0370 gr<br>0.179 OD       | 1(+)<br>0.0369 gr<br>0.163 OD        | 1(+)<br>0.0352 gr<br>0.338 OD       | 1(+)<br>0.0339 gr<br>0.278 OD        | 1(+)<br>0.0411 gr<br>0.271 OD        | 1(+)<br>0.0218 gr<br>0.095 OD        |
| 2(+)<br>0.0393 gr<br>0.126 OD        | 2(+)<br>0.0403 gr<br>0.274 OD      | 2(+)<br>0.0350 gr<br>0.176 OD       | 2(+)<br>0.0343 gr<br>0.275 OD        | 2(+)<br>0.0271 gr<br>0.151 OD       | 2(+)<br>0.0370 gr<br>0.374 OD        | 2(+)<br>0.0335 gr<br>0.210 OD        | 2(+)<br>0.0207 gr<br>0.148 OD        |
| 3(+)<br>0.0334 gr<br>0.202 OD        | 3(+)<br>0.0371 gr<br>0.203 OD      | 3(+)<br>0.0373 gr<br>0.208 OD       | 3(+)<br>0.0371 gr<br>0.177 OD        | 3(+)<br>0.0350 gr<br>0.187 OD       | 3(+)<br>0.0376 gr<br>0.318 OD        | 3(+)<br>0.0266 gr<br>0.182 OD        | 3(+)<br>0.0214 gr<br>0.137 OD        |
| 4(+)<br>0.0392 gr<br>0.292 OD        | 4(+)<br>0.0407 gr<br>0.142 OD      | 4(+)<br>0.0406 gr<br>0.237 OD       | 4(+)<br>0.0361 gr<br>0.178 OD        | 4(+)<br>0.0339 gr<br>0.244 OD       | 4(+)<br>0.0289 gr<br>0.422 OD        | 4(+)<br>0.0298 gr<br>0.161 OD        | 4(+)<br>0.0241 gr<br>0.287 OD        |
| 5(-)<br>0.0314 gr<br>0.241 OD        | 5(-)<br>0.0326 gr<br>0.101 OD      | 5(-)<br>0.0274 gr<br>0.071 OD       | 5(-)<br>0.0392 gr<br>0.315 OD        | 5(-)<br>0.0296 gr<br>0.191 OD       | 5(-)<br>0.0378 gr<br>0.240 OD        | 5(-)<br>0.0334 gr<br>0.219 OD        | 5(-)<br>0.0247 gr<br>0.080 OD        |
| 6(-)<br>0.0341 gr<br>0.260 OD        | 6(-)<br>0.0291 gr<br>0.091 OD      | 6(-)<br>0.0312 gr<br>0.063 OD       | 6(-)<br>0.0350 gr<br>0.302 OD        | 6(-)<br>0.0330 gr<br>0.209 OD       | 6(-)<br>0.0318 gr<br>0.188 OD        | 6(-)<br>0.0247 gr<br>0.183 OD        | 6(-)<br>0.0266 gr<br>0.187 OD        |
| $I_{V(+)}=23.05$<br>$I_{V(-)}=15.29$ | $I_{V(+)}=27.7$<br>$I_{V(-)}=6.22$ | $I_{V(+)}=21.25$<br>$I_{V(-)}=4.60$ | $I_{V(+)}=22.12$<br>$I_{V(-)}=16.65$ | $I_{V(+)}=27.7$<br>$I_{V(-)}=12.78$ | $I_{V(+)}=47.69$<br>$I_{V(-)}=12.25$ | $I_{V(+)}=25.09$<br>$I_{V(-)}=13.95$ | $I_{V(+)}=29.79$<br>$I_{V(-)}=10.26$ |

The percentage of cell viability was calculated on viability index  $I_v (+)$ . To evaluate the efficacy of each decellularization method, we analyzed the percentage of cell viability in fresh tissue compared to the corresponding ADM (**Fig. 3, 5B, 6B, 7B, 8B**). Moreover, we evaluated the intrinsic variability of tissue samples comparing different fresh tissues and ADM treated with the same decellularization protocol (**Fig. 3, 5B, 6B, 7B, 8B**). Results show that all decellularization protocols drastically reduced the percentage of cell viability in ADM samples (**Fig. 3, 5B, 6B, 7B, 8B**). In particular, the decellularization obtained by protocol 4 was more effective than others (**Fig. 8B**). We also identified that the percentage of cell viability of different fresh tissues and ADM treated with the same protocol yield comparable results (**Fig. 5B, 6B, 7B, 8B**). Thus, the intrinsic variability of tissues does not affect the tissue decellularization.

To analyze the decellularization effects on tissue morphology and structure, histological examination of ADM was performed using Hematoxylin and Eosin staining. Fresh tissues were used as controls. Results showed that ADM matrices revealed some common features in comparison with controls such as normal architecture and the preservation of large amounts of collagen fibers. However, in the ADM matrices, there was a drastic reduction in visible cells with signs of necrosis and degeneration of fibroblast nuclei as well as an absence of tissue edema (**Fig. 5A, 6A, 7A, 8A**).

## Discussion

The use of circumcision for the treatment of clinical conditions such as redundant foreskin and phimosis is well established (Wu et al., 2013; Hayashi and Kohri, 2013). Other indications for circumcision are more contentious (Larke et al., 2011; Frisch et al., 2013). Most circumcisions are performed for non-therapeutic reasons despite the fact that no medical organization recommends it and several national medical organizations have condemned the practice. The effects of this procedure on sexual function have been widely debated and the opinions about its therapeutic use are still controversial.

Among the disadvantages identified by circumcised men, a reduction of penile sensitivity has been recently confirmed (Bronselaer et al., 2013). Removing the foreskin has consistently been associated with a decrease in sexual pleasure. After circumcision, the uncovered glans became thicker and dryer since it is continuously submitted to friction and irritation that, in turn, promote the mucosa keratinization, leading to a loss of sensation. When considering the psychological consequences, the procedure in an infant is painful enough to have a psychological impact that increases irritability and interferes with infant-maternal interaction (Goldman, 1999). For these reasons, the application of non-therapeutic circumcision should be restricted only to adults after they have provided fully-informed consent.

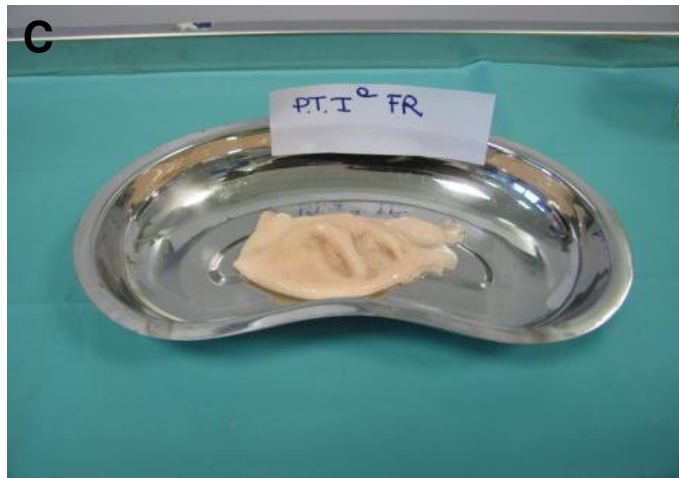
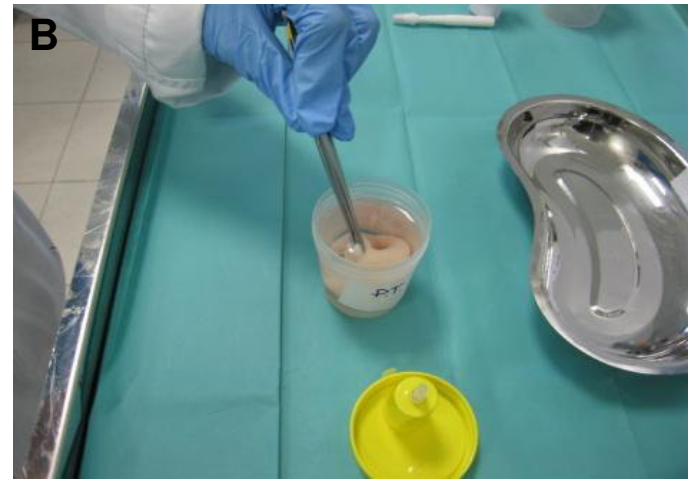
In agreement with this idea, Foregen onlus seeks to support those suffering with the physical and psychological problems reported in circumcised men. Foregen onlus is attempting to offer a solution through the use of innovative approaches through regenerative medicine.

In this study we present preliminary data for the development of an *in vitro* decellularization method based on the use of animal derived membrane (ADM) obtained from bull foreskin as a starting point to provide an effective, regenerative solution to the damages of circumcision.

Although these preliminary data need to be improved before implementing therapeutic use of the developed decellularization method, the drastic reduction of cell viability observed in all ADM obtained in our study appears promising. To ensure the reproducibility of the results, each protocol was performed on two different foreskin tissues derived from different bulls to account for the tissue's intrinsic variability. In particular, protocol 4 showed the best capacity to minimize cell viability.

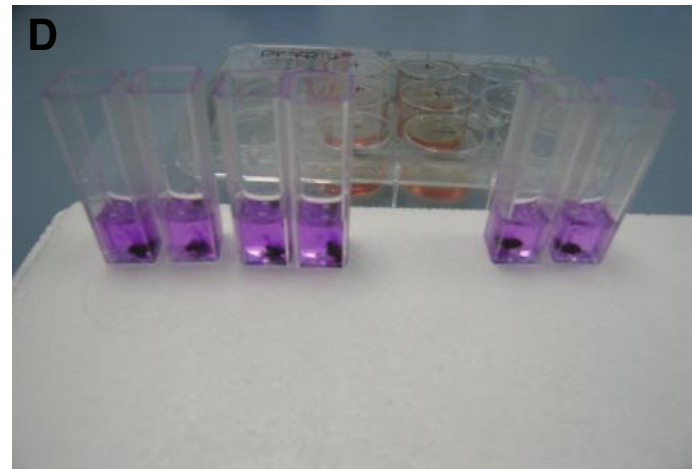
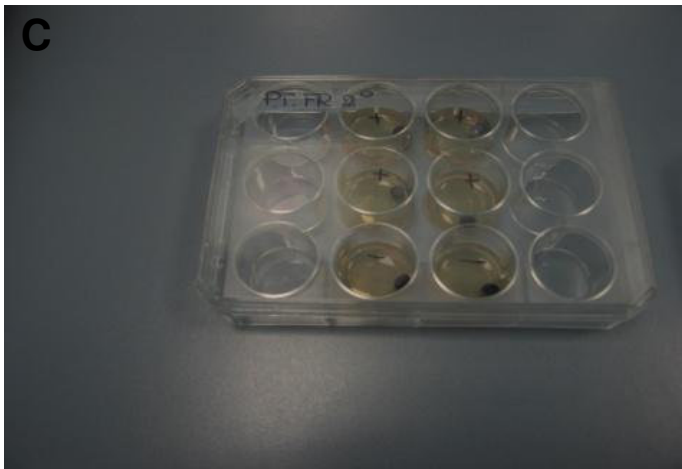
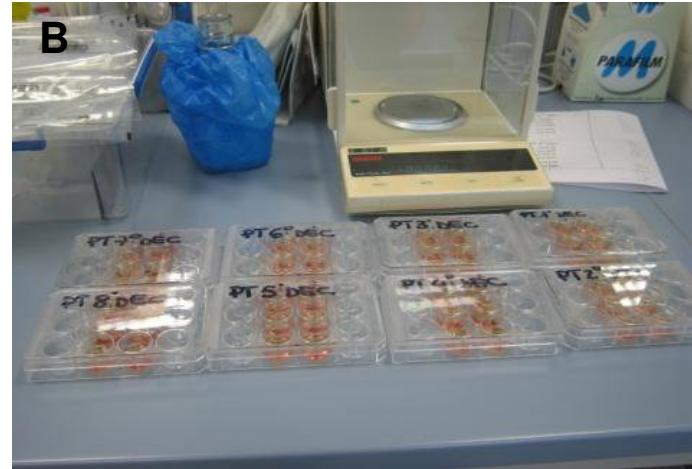
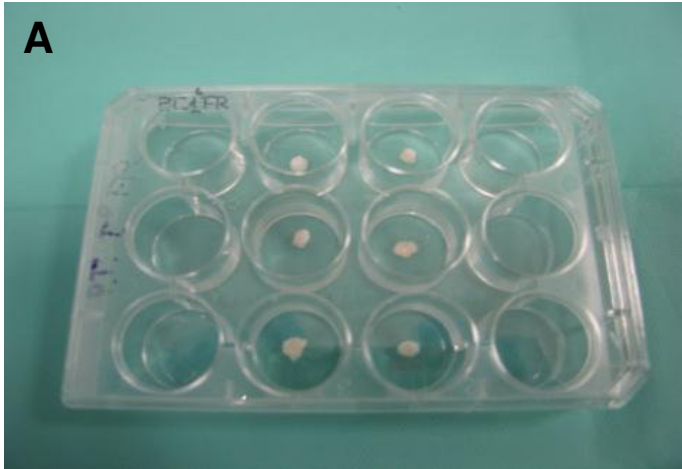
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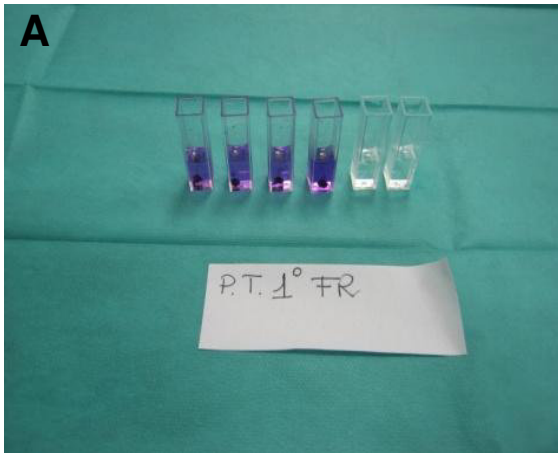
**Fig 1**



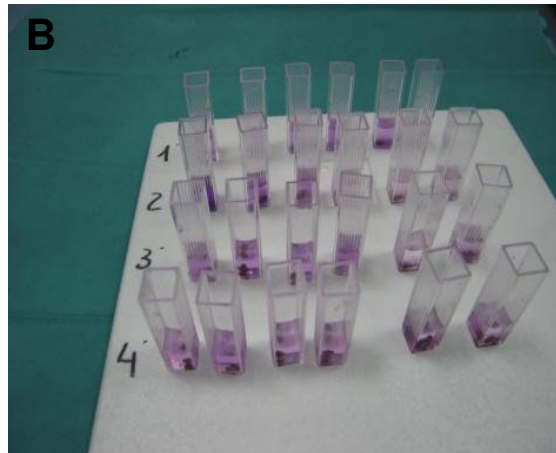


**Fig 2**

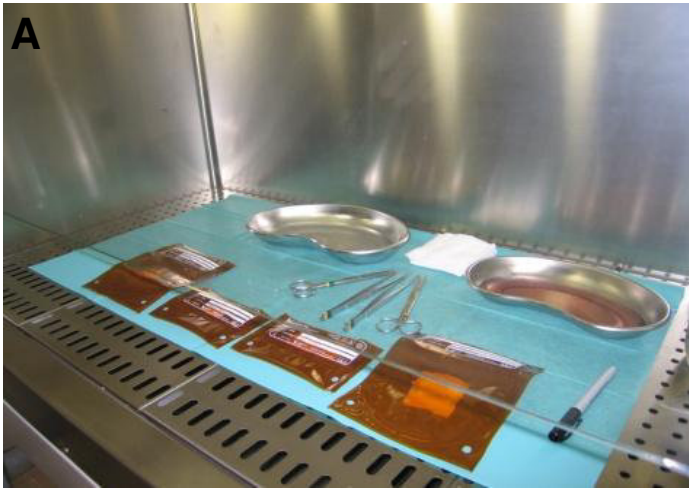
**FRESH TISSUE**



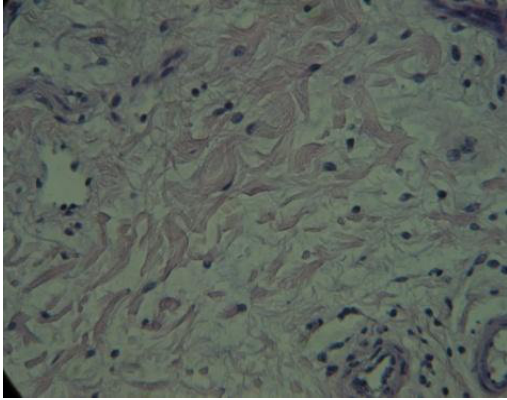
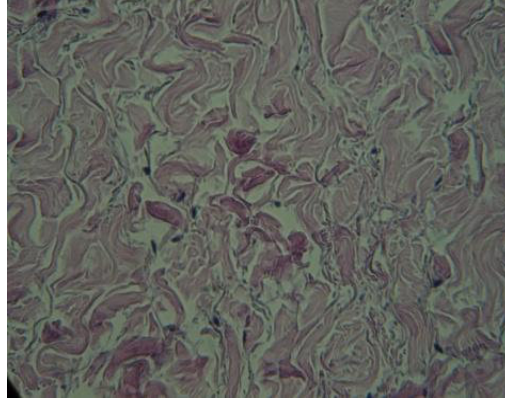
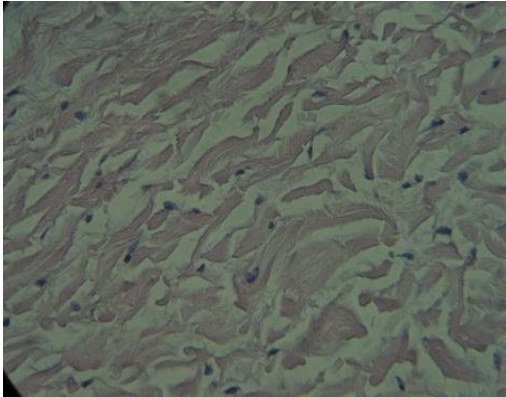
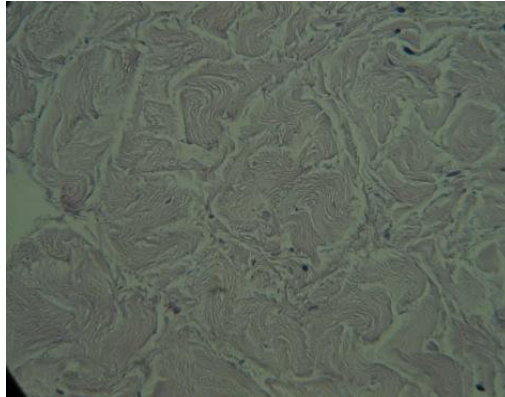
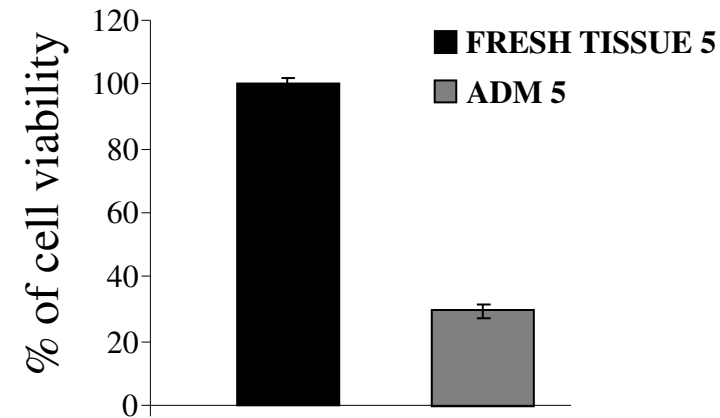
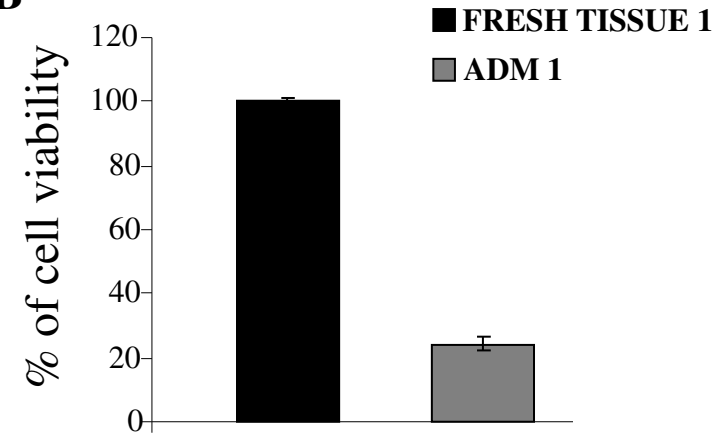
**ADM**



**Fig 3**

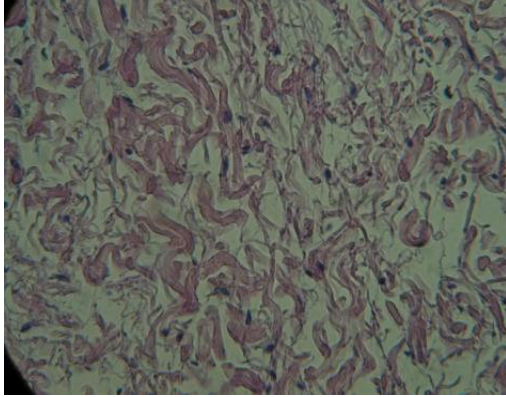
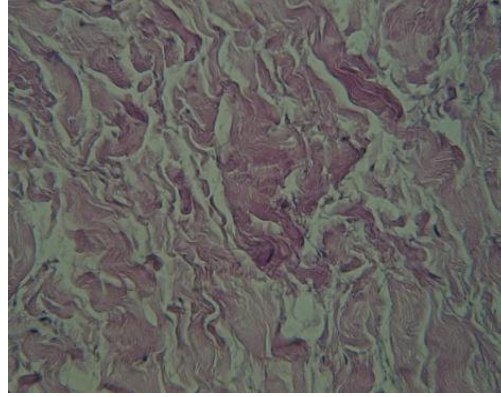
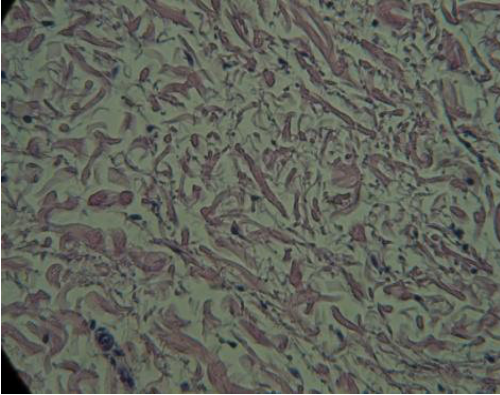
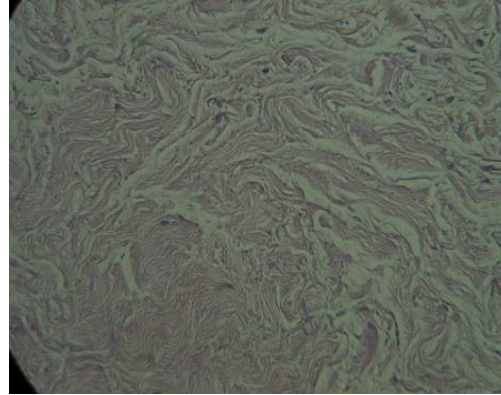
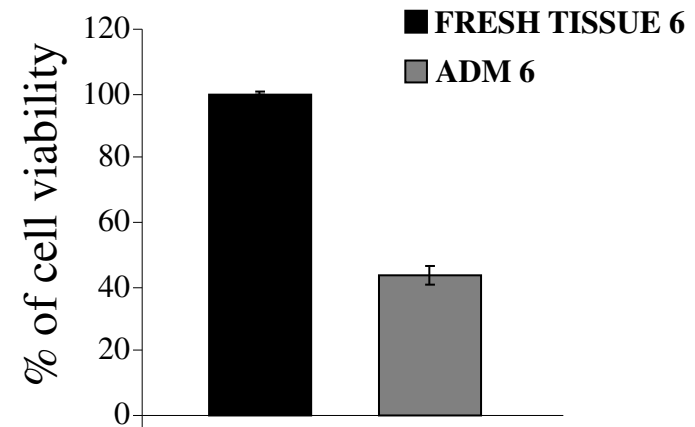
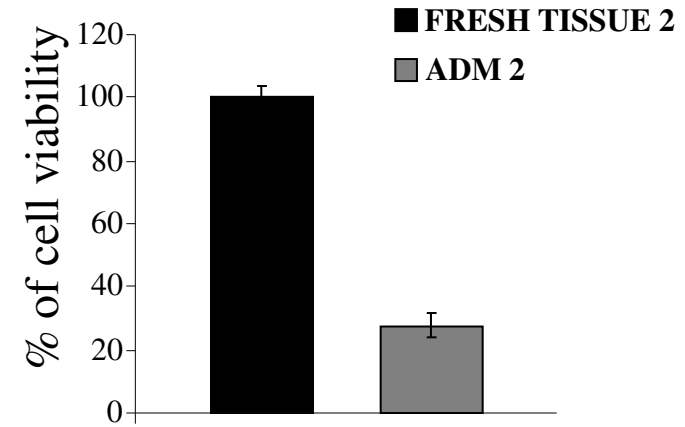


**Fig 4**

**A****FRESH TISSUE 1****ADM 1****FRESH TISSUE 5****ADM 5****B****Protocol 1**Pre-freezing at  $-80^{\circ}\text{C}$ 

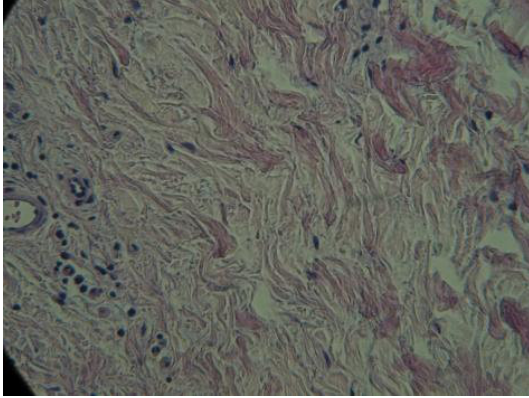
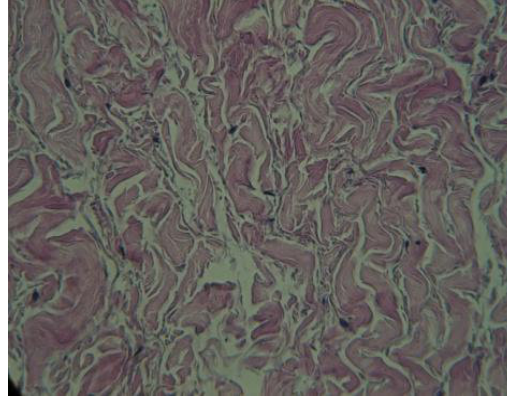
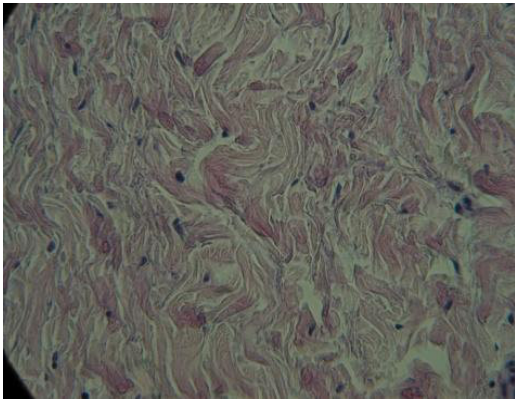
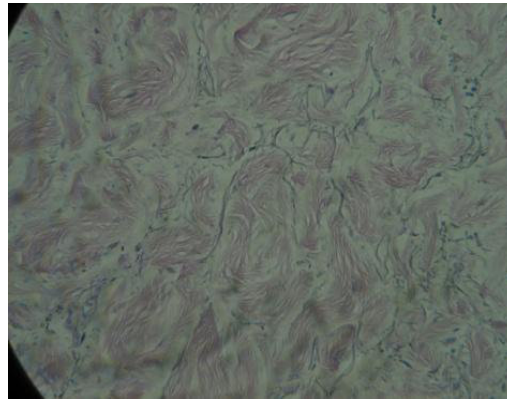
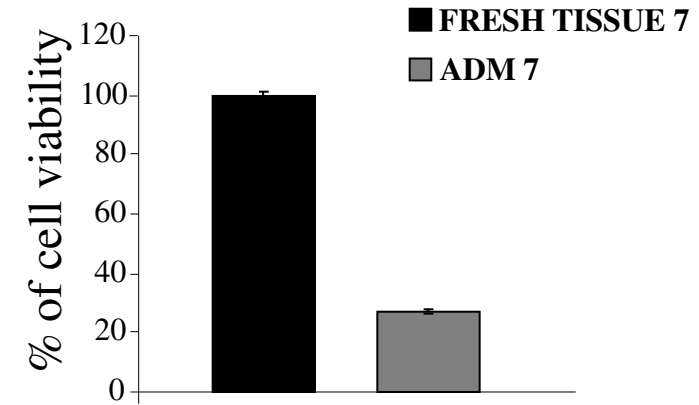
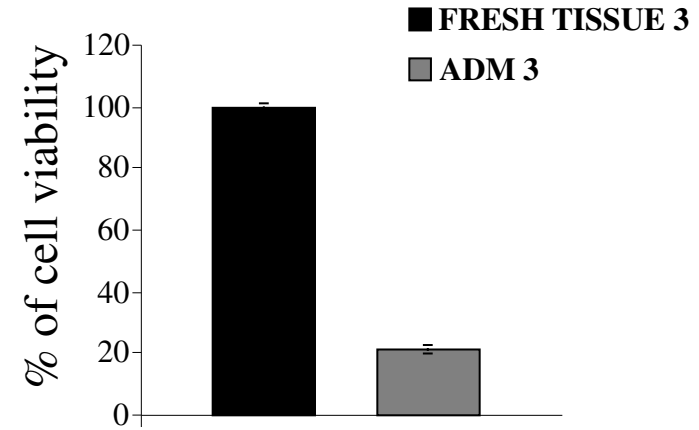
Trypsin 1X for 48h

Freezing at  $-80^{\circ}\text{C}$ **Fig 5**

**A****FRESH TISSUE 2****ADM 2****FRESH TISSUE 6****ADM 6****B**

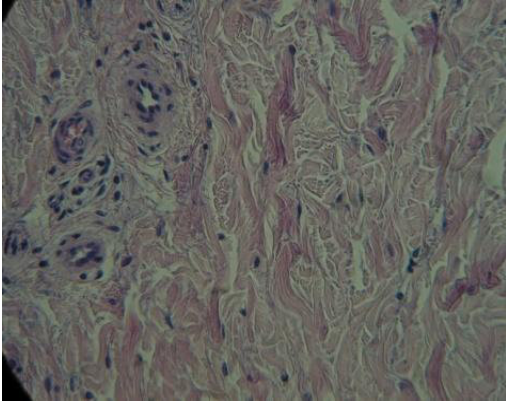
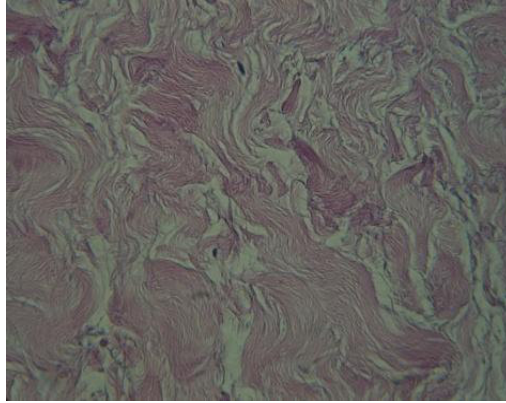
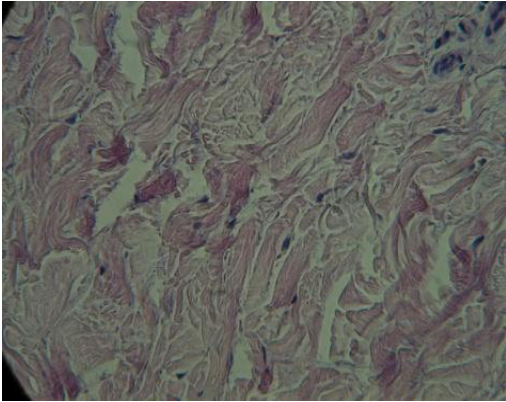
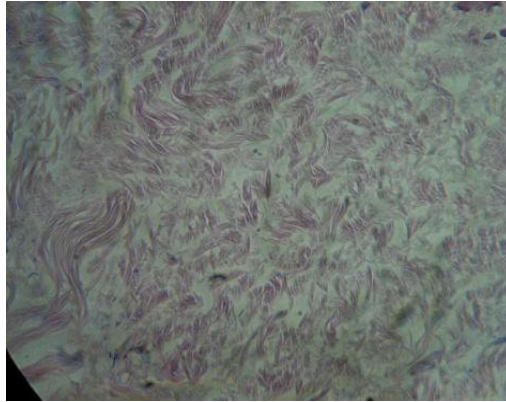
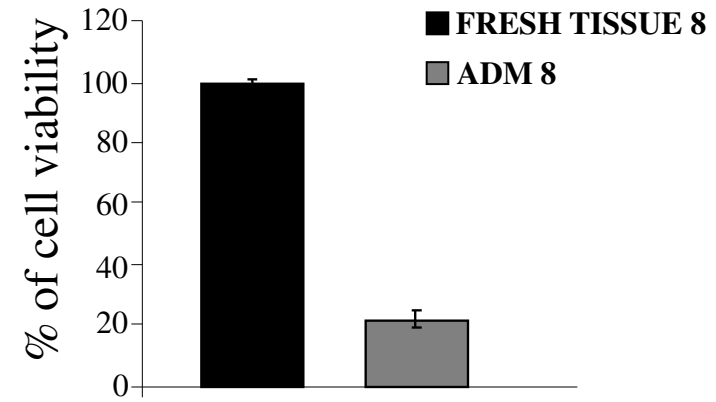
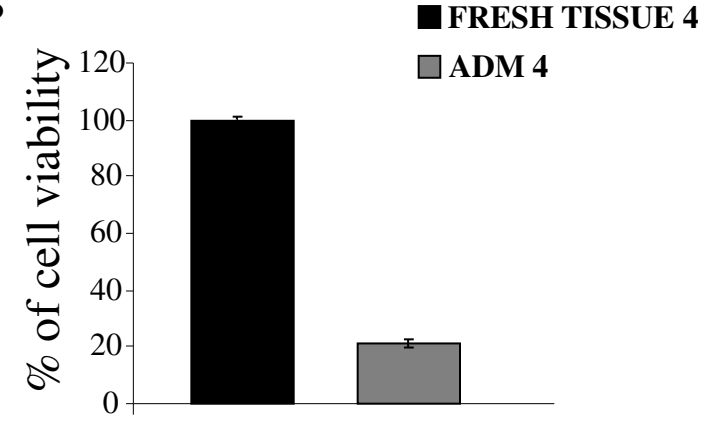
**Protocol 2**  
Trypsin 2X for 24h  
Freezing at -80°C

**Fig 6**

**A****FRESH TISSUE 3****ADM 3****FRESH TISSUE 7****ADM 7****B**

**Protocol 3**  
Trypsin 3X for 24h  
Freezing at -80°C

**Fig 7**

**A****FRESH TISSUE 4****ADM 4****FRESH TISSUE 8****ADM 8****B**

**Protocol 4**  
Trypsin 4X for 24h  
Freezing at -80°C

**Fig 8**