Acellular adipose matrix as a natural scaffold for tissue engineering

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Summary

Background: In conventional tissue-regeneration technologies, stem cells and/or other cells are injected into or incubated on scaffolds. In general, scaffolds can be classified into synthetic and natural polymers and natural matrices. Polymers are generally less suitable than natural matrices in terms of biocompatibility and biodegradability. A highly promising alternative may be the acellular adipose matrix (AAM), which is a natural scaffold that could mediate tissue regeneration without any artefacts. The optimal method for adipose-tissue decellularisation is described in this article.

Methods: Discarded human adipose tissues harvested from routine operations were used. In experiment 1, four different adipose-tissue-decellularisation methods were compared and modified. In experiment 2, the most effective method was tested by using adipose-tissue blocks from various donor sites (the abdomen, chest and forearm) and of different weights (0.8, 25 and 80 g). Haematoxylin and eosin (H&E) staining, immunohistochemistry (IHC) and scanning electron microscopy were used to determine the efficacy of decellularisation.

Results: In experiment 1, a method using an enzymatic digestion solution yielded complete decellularisation after some modifications. In experiment 2, the 0.8-g specimens were completely decellularised by the modified method. However, cell components remained in the 25- and 80-g specimens. The donor site had no effect on the degree of decellularisation.

Conclusions: An optimal method for adipose-tissue decellularisation is reported. Because AAM is a natural collagen scaffold that is of human origin, this report describes an
Autologous tissue transfers or synthetic materials are generally used to repair tissue defects.\(^1\) Despite improvements in operative techniques, invasiveness and complications such as necrosis, infections, shrinkage and oil-cyst formation can result in serious problems.\(^2\) Moreover, the long-term survival and functionality of the transplanted materials can be limited. Synthetic and non-human biologically derived materials that have been used as injectable tissue fillers, along with volume-filling constructs that are used for soft-tissue repair, have the advantage of not requiring donor-site sacrifice. However, transfer of these materials often results in infection, resorption or foreign-body responses.\(^3\) An ideal alternative would be a regenerative method that involves the harvesting of human tissue with low invasiveness and that permits the reuse of these tissues.

A highly attractive regenerative technique may be to use acellular adipose tissue matrix (AAM). Since the AAM is composed of collagen, it may be suitable for the regeneration of various organs. Moreover, like adipose-stem-cell transfer, which has attracted a great deal of attention in the tissue regeneration field recently, the great advantage of this matrix is that its harvest requires little donor-site sacrifice. In the present article, several different decellularisation methods that have been described previously were compared in terms of their ability to decellularise adipose tissue. After certain modifications, a highly effective method was obtained. This method was then tested with adipose-tissue specimens of different sizes and from different parts of the body.

Materials and methods

In accordance with the regulations set forth by the Institutional Review Board (IRB) of Nippon Medical School Hospital, informed consent was obtained from all patients and their families. Discarded human adipose tissues harvested from routine operations were used. The samples were delivered in sterile physiological saline to the lab for processing within 2 h.

Experiment 1: comparison and modification of different decellularisation methods

Prior to processing, the adipose tissue samples were cut into blocks whose masses ranged from 0.7 to 0.8 g. This weight was selected according to the previously published papers. Four decellularisation methods that have been described previously were selected.\(^4^-^8\) These methods were originally established to decellularise different tissues. Various steps in each method were modified, as shown in Table 1, and the abilities of these modified methods to decellularise adipose tissue were analysed. The most effective method was selected as follows.

Method A was developed for muscle decellularisation.\(^4\) This method was selected because of its feasibility and cost-effectiveness. The original method required that the tissue be subjected to three cycles of freeze-thawing using liquid nitrogen. The cycle number was modified from 3 to 6 (Modified A-1), 12 (Modified A-2) and 18 (Modified A-3) (Table 1).

Method B was developed to isolate intact basement membranes from bovine retinal and brain blood vessels, rabbit renal tubules and rat renal glomeruli.\(^5\) This method was selected because it is a classical method that has been widely adapted to many other organs with several modifications.\(^6\) Thus, the tissues were first placed in distilled water containing 0.1% sodium azide for 1 h (step 1). The tissue suspension was then centrifuged and the pellet was suspended and incubated for 1 h in 40 ml of 1 M NaCl (Wako Chemical, Tokyo, Japan) and 2000 Kunitz units of DNase (Sigma, Deoxyribonuclease 1) (step 2). The mixture was centrifuged again and the pellet was suspended and incubated for 2 h in 40 ml of 4% sodium deoxycholate containing 0.1% sodium azide (step 3). Finally, the mixture was centrifuged and rinsed. Method B was modified by lengthening incubation steps 1, 2 and 3 by two- (Modified B-1), four- (Modified B-2) and eightfold (Modified B-3) (Table 1), respectively.

Method C was developed to decellularise embryoid bodies.\(^7\) The method was selected because of its feasibility. Thus, the tissue was kept in a serum-free media for 3 h,
after which the tissue was centrifuged and placed in Triton X-10 (VWR) for 1 h (step 1). The mixture was centrifuged again and the pellet was suspended in 2000 Kunitz units of DNase (Sigma, Deoxyribonuclease 1) and stirred for 1 h (step 2). At the end of the treatment, the mixture was centrifuged and rinsed. Method C was modified by lengthening incubation steps 1 and 2, from 1 h and 1 h to 4 h and 8 h (Modified C-1) and 16 h and 24 h, respectively (Modified C-2) (Table 1).

Method D was developed for adipose-tissue decellularisation. The tissues were first subjected to three cycles of freeze-thawing in a freezing buffer solution, which is a hypotonic Tris buffer (pH 8.0) containing 10 mM Tris base and 5 mM ethylenediaminetetraacetic acid (EDTA) (Gibco, Burlington, Canada). Next, the tissues were incubated for 16 h in enzymatic digestion solution 1, which consisted of 0.25% trypsin/0.1% EDTA (step 1). The samples then underwent a 48-h polar solvent extraction in 99.9% isopropanol (Wako Chemical, Tokyo, Japan). Thereafter, the processed tissues were rinsed and incubated for another 6 h in enzymatic digestion solution 1 (step 2). After three more washes, the tissues were incubated for 16 h in enzymatic digestion solution 2, which consisted of 55 mM Na2HPO4, 17 mM KH2PO4, 4.9 mM MgSO4, 7H2O, 15,000 U DNase type II (from bovine pancreas), 12.5 mg RNase type III A (ribonuclease; from bovine pancreas), and 2000 U lipase type VI-S (from porcine pancreas) (step 3). The tissues were then rinsed and subjected to a final polar solvent extraction in 99.9% isopropanol for 8 h. At the end, the tissues were rinsed again. This protocol was modified by lengthening steps 1, 2 and 3 from 16 h, 6 h and 16 h to 16 h, 20 h and 30 h, respectively (Modified D-1) (Table 1).

Experiment 2: optimisation of the selected method

The most efficacious method for decellularisation of the adipose tissue was selected at the end of experiment 1. The aim of experiment 2 was to determine the optimum weight that can be sufficiently decellularised using the selected method and also to find out if there is any difference between the adipose tissues from different donor sites. Therefore, the selected method was tested with 0.8 g of adipose tissue from abdomen, chest and forearm and with different weights of adipose tissue from the abdomen (0.8, 25 and 80 g).

Histology

Haematoxylin and eosin and immunohistochemical staining

Representative samples of unprocessed human adipose tissue and tissue after each procedure were fixed in formalin, embedded in paraffin, sectioned into 5-µm slices and affixed to glass slides. Haematoxylin and eosin (H&E) staining was used to detect the presence of residual cells or cell fragments in experiments 1 and 2. Immunohistochemical (IHC) staining for collagen type IV and laminin was conducted to assess the architecture of the basement-membrane components in experiment 2. The antibodies that were used were rabbit polyclonal anti-collagen type IV antibody (1:100; AbCam, Cambridge, MA, USA) and rabbit polyclonal anti-laminin antibody (1:100; AbCam, Cambridge, MA, USA). Antibody binding was detected by using the VECTASTAIN Elite ABC rabbit IgG kit (Vector laboratories, Burlingame, CA, USA). The VECTOR® NovaRED™ substrate kit (for peroxidase) (Vector laboratories, Burlingame, CA, USA) served as the enzyme substrate.

Transmission electron microscope

Transmission electron microscopy was conducted on AAM samples isolated from various regions and on different sizes of AAM isolated from the abdominal region in experiment 2 to confirm decellularisation and to assess the architecture of the matrix. Oolong tea extract (OTE) staining was used for the ultrastructural observation of connective tissues. Thus, the tissue was cut into small pieces and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at room temperature for 2 h. The specimens were then postfixed in 1% osmium tetroxide, dehydrated in a graded series of alcohol and embedded in Epok 812. Serial ultrathin sections were cut by a Leica ultracut F ultramicrotome (Leica, Wetzlar, Germany) with a diamond knife and picked up on copper grids. The grids were stained with 0.2% OTE (OTE powder; Suntory, Osaka, Japan) in 0.1 M phosphate buffer. The sections were soaked in single drops of the OTE solution for 20–30 min at room temperature. The sections were then submerged in single drops of distilled water for 5 min, post-stained with uranyl acetate and lead citrate, and examined at 80 kV under a H-7500 electron microscope (Hitachi, Tokyo, Japan).

Results

Experiment 1

All the results were compared to unprocessed adipose tissue histologically (Figure 1a). After method A, the cells and cell components remained, even when the freeze–thaw cycles were increased by sixfold from 3 to 18 (Figure 1b). Moreover, the macroscopic view revealed that the matrix structures were destroyed. After methods B and C, cells or cell fragments continued to be observed, especially in the vessel walls. This was true even when the incubation times of the various steps were lengthened by at least eightfold (Figure 1c, d). When the original protocol of method D was followed, cells and cellular components also remained (Figure 2a). However, complete decellularisation was achieved when some modifications were instituted (Figure 2b). As shown in Figure 3, a significant volume of loose, white matrix (designated AAM) remained after the 0.8-g tissue was processed by modified method D-1. The AAM had similar dimensions to the tissue block at the beginning of the processing, which suggested that the architecture of the matrix was well preserved. The hydrated mass of the AAM represented between 30% and 40% of the original tissue mass.

H&E staining confirmed the absence of cells and cell debris in the AAM.

To assess the architecture of the basement components in the AAM, IHC staining of collagen type IV and laminin
was conducted. In human adipose tissue, laminin and collagen IV are expressed in the basement membrane that separates individual adipocytes and the lining of the supporting vascular structures. The IHC-staining analysis revealed that the laminin and collagen IV contents were preserved during the tissue processing (Figure 4). Regarding the staining patterns, laminin and collagen IV lined the decellularised, empty lumens of former blood vessels. Thus, the vascular structures were conserved during the processing.

TEM analysis revealed the absence of cells or cell fragments and that the ultrastructure of the collagen fibrils (CFs) of the processed matrix were well preserved (Figure 5).

The pore size of the resulting AAM was found to be between 20 and 100 μm in diameter. The modified method D-1 was thus selected for further testing.

Experiment 2

Subsequently, 0.8-g adipose-tissue blocks from various donor sites, namely, the abdomen, chest and forearm, were processed by modified method D-1. H&E staining revealed that all samples lacked cells and cell debris (Figure 6). Thus, no difference was found between the adipose tissues from different donor sites in terms of the efficacy of modified method D-1.

Adipose tissues with different weights, namely, 0.8, 25 and 80 g, were then processed by modified method D-1. As expected, H&E staining revealed that the 0.8-g specimens were completely decellularised (Figure 7a). Although the periphery of the 25-g specimens was decellularised (Figure 7b), cell components remained in the central area (Figure 7c). Moreover, in 80-g specimens, cell components remained both in the periphery (Figure 7d) and in the
central area (Figure 7e). Therefore, the optimal weight of adipose tissue for preparation of AAM using the selected method was determined to be 0.8 g.

Discussion

Tissue-engineered adipose tissue may be suitable for improving soft-tissue volume and shape in both reconstructive and cosmetic applications.10,11 Since the adipose scaffold that we describe in this paper is acellular, it does not contain any immunogenic components; therefore, it can easily be transplanted to other patients without the risk of immune rejection. Significant quantities of human adipose tissue are routinely discarded as medical waste during routine operations. Discarded fat tissue can be stored and used as a source for acellular adipose scaffold on demand. Even though the initial operations on the donors may involve large donor-site scars, the insertion of the preserved, non-immunogenic acellular adipose scaffold into the body of other patients will prevent the creation of new donor-site scars in the body of recipients. Conventional tissue-regeneration technologies involve the injection or incubation of stem cells and/or other cells in scaffolds that range from synthetic or natural polymers to natural matrices.12 Since synthetic and natural polymers have limitations in terms of their biocompatibility and biodegradability, natural matrices are a more promising option for tissue regeneration. Most soft- and hard-connective tissues, such as bone, cartilage, tendon, cornea, blood vessels and skin, contain CF that are arranged around the cells in a three-dimensional network called the extracellular matrix (ECM). Collagen, the main component of ECM, is the most abundant protein in mammals,13 making up

Figure 3  Macroscopic findings of a 0.8 g adipose tissue block before (left) and after (right) processing by modified method D-1.

Figure 4  IHC staining of normal human adipose tissue and the AAM. Immunostaining for laminin (a&c) and collagen type IV (b&d) showed that both basement membrane components localised along the lumens of the vascular structures were preserved throughout the decellularisation process. Scale bars = 100 μm.
approximately 25–35% of the whole-body protein content. The ECM not only offers structural support for cells but also profoundly influences the major cellular programs of growth, differentiation and apoptosis. These properties mean that animal-derived collagens are one of the most useful biomaterials available. Indeed, they are widely used for tissue engineering. However, the use of these animal-derived collagens, including bovine and porcine collagen, in humans can be compromised by immune reactions/sensitisation and the possibility of xenogenic disease transmission. Alternative biomaterials should mimic both the physical and biochemical properties of native collagens. It is a well-known fact that the tissues from the same species reduce the risk of an immune reaction, as well as xenogenic disease transmission, typically associated with the use of animal products, such as bovine or porcine collagen. This means that AAM may be ideal: as an autologous collagenous matrix, it can be used for adipose-tissue regeneration with minimal concerns about allergic reactions and pathogen transmission. Furthermore, the preserved vascular architecture can be of benefit in terms of promoting the organisation of infiltrating endothelial cells to vascularise the construct and allow for vascular ingrowth, subsequently improving the viability of the transplanted grafts. Thus, the structure of human origin scaffold may provide an ideal environment for human cells to grow with its preserved three-dimensional structure and vascular network.

When 0.8-g adipose-tissue specimens were processed by using modified method D-1, between 30% and 40% of the original adipose-tissue weight remained. The pore size was then estimated because biomaterials that must replace native collagen-based ECM should have adequate scaffold porosity. This is needed to facilitate cell migration and the growth of blood vessels across the scaffold and to ensure the effective exchange of nutrients and waste products between the cells and their microenvironment. It has been suggested that the ideal pore size is between 10 and 100 μm. AAM has pore sizes between 20 and 100 μm, which means that it can function as a scaffold. The cellularity and cell size of adipose tissue is known to differ depending on the region of the body, sex, age and body conditions, such as metabolic abnormalities and obesity. However, when 0.8-g adipose-tissue blocks from different donor sites were processed in the present study, differences between donor sites were not observed. Adipose-tissue blocks that differed in weight were also processed. While the 0.8-g specimens were completely acellular, the 25- and 80-g blocks continued to bear cells. Thus, modified method D-1 is suitable for adipose tissue from various regions of the body as long as the specimens are about 0.8 g in weight. Further research is needed to develop a protocol that will completely decellularise larger tissue specimens. To date, 28 collagen types have been identified. Types I, II, III and V are the main ones that make up the essential collagen in bone, cartilage, tendon, skin and muscle. Adipose-tissue ECM also contains multiple types of collagen, including I–VI. Adipose tissue is histologically categorised as a type of loose connective tissue, and collagen contributes considerably to the non-cell mass of this tissue. Moreover, each adipocyte is surrounded by a thick ECM, referred to as basal lamina, of which the main component is collagen. Since osteoblasts and chondrocytes, like adipocytes, are of mesenchymal origin, the basal lamina characteristic of adipocytes is shared by these bone and cartilage cells. It would be ideal to use human origin scaffolds for bone and cartilage regeneration for the same reasons that human adipose-tissue-derived scaffolds would be ideal for adipose-tissue regeneration. However, the use of human origin scaffolds in bone and cartilage regeneration has not been reported previously. This may reflect the significant difficulties that are faced in generating sufficient amounts of bone or cartilage-derived scaffold material. By contrast, significant quantities of human adipose tissue are routinely discarded after abdominoplasty, liposuction or panniculectomy. We assume that AAM could be stored in a freezer of freeze and dry...
Since the structure of adipose tissue is similar to bone and cartilage tissue, AAM may also be suitable as a scaffold for bone or cartilage regeneration. It is also possible that injections with minced AAM could serve as a non-absorbing natural filler in the cosmetic field. Further studies on the function and utility of this material are needed to determine its suitability in the clinic.

Conclusions

An optimal method for decellularising adipose tissue is described here. Since the AAM scaffold is a natural collagen scaffold and is derived from humans, it can be used for clinical application with minimal risks. Its preserved three-dimensional structure and the intact vascular network should greatly enhance the cell–cell and cell–matrix interactions within the scaffold upon cell seeding and in vivo transplantation, subsequently yielding a better viability. This report describes an important first step in a tissue-engineering innovation that may be suitable for the regeneration of different tissues.

Conflict of interest/funding statement

Authors have no competing financial interests or funding to declare.

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