Human decellularized adipose tissue hydrogels as a culture platform for human adipose-derived stem cell delivery

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Abstract
Adipose-derived stem cells (ADSCs) have been widely researched and used as a drug therapy in many fields like disease treatment and tissue engineering. However, ADSCs are susceptible to the surrounding environment. The emergence of acellular extracellular matrix provides a solution, which can serve as biomaterial scaffold as well as original ecological niche for the stem cells. Therefore, we propose the hypothesis that human decellularized adipose tissues (hDAT) are processed into injectable hydrogels and then mixed evenly with ADSCs. So that the ADSCs embedded-hydrogels could directly carry the stem cells to the appropriate sites. The hDAT hydrogel could provide microenvironmental protection for ADSCs. In this study, we successfully made human decellularized adipose tissue hydrogel (hDAT-gel), which was temperature-sensitive, liquid at 4°C and semi-solid at 37°C. When the ADSCs were embedded in hDAT-gel, they survived well and continued to grow well in layers. When the pre-gel containing ADSCs was injected subcutaneously into nude mice, the sample results after 15 min showed gelation occurred in situ. These results suggested that hDAT-gel could provide a culture platform for ADSCs delivery.

Keywords
Adipose-derived stem cells, human decellularized adipose tissue, injectable hydrogel, cell delivery, extracellular matrix

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Introduction
Extracellular matrix (ECM) is a complex network structure composed of macromolecules secreted by cells into stroma. ECM not only statically performs physical functions such as support, connection, water retention, protection and compression resistance, but also plays an all-round biological role in the basic activities of cells. Acellular extracellular matrix is the natural tissue from which cells and antigen composition are removed, retaining the original specific structure. A variety of decellularized tissues have become popular biomaterial scaffold on account of their low immunogenicity and good biocompatibility.1–5 Dermis, small intestinal submucosa and bladder can be processed into various shapes after acellular treatment. After a sterile treatment, they can be surgically transplanted to the corresponding defect site for tissue regeneration and repair.6–8 In addition, acellular extracellular matrix can recombine stem cells.9,10 What is more, they can be further made into injectable hydrogels.11–14

In the field of plastic surgery, adipose tissue is widely used for contour sculpture, breast augmentation and so on. Much of adipose tissue after liposuction is discarded as...
medical waste. On one hand, as connective tissue, adipose tissue are rich in ECM. On the other hand, as endocrine tissue, adipose tissue can secrete numerous cellular active factors into ECM. It is one of the most abundant, consumable and easily harvested biomaterials. In recent years, many scholars have focused on the application of decellularized adipose tissues as scaffold materials for fat, cartilage or bone tissue regeneration. Furthermore, the hydrogel form of acellular ECM has also attracted much attention from researchers. The hydrogel form offers the possibility of minimally invasive application in the future. Many acellular ECM hydrogels have been extracted from multiple tissues, some of which have already in clinical trials. For instance, porcine myocardial acellular matrix are highly susceptible to external influences. In this study, we transformed human adipose acellular matrix into the form of injectable hydrogel, which is thermosensitive. The hDAT-gel was used as culture platform for ADSCs, and provided an ecological niche for the stem cells. We believe this would furnish some practice basis for the further clinical application of ADSCs-embedded hDAT-gel.

**Materials and methods**

**Materials**

The adipose tissue used in the study came from patients who underwent liposuction in the Department of Plastic and Reconstructive Surgery, the First Medical Center, Chinese PLA (People’s Liberation Army) General Hospital. Under normal procedures, adipose tissue derived from liposuction is treated as medical waste. The sample collection for this study and all animal experiments were reviewed and approved by Institutional Review Board of Chinese PLA (People’s Liberation Army) General Hospital.

**Preparation of hDAT-gel**

Firstly, we conducted decellularization of adipose tissue according to the protocol published in *Methods in Molecular Biology* by Flynn et al. Cells and lipid components in native adipose tissue were removed by the comprehensive treatment of trypsin, isopropanol and three enzymes (deoxyribonuclease, ribonuclease and lipase). Then we put the decellularized adipose tissue into a lyophilizer (Gamma 2–16, Christ) to dry completely. Next, the lyophilized acellular adipose tissue was cut into pieces and ground into powder by grinding mill (KZ-II, Servicebio) after quick-freezing in liquid nitrogen. Then the decellularized adipose tissue lyophilized powder collected through a 40-mesh screen was digested at 10 mg/mL in 0.01 M HCl using 1 mg/mL pepsin (250 U/mg, Sigma-Aldrich), which was stirred at a constant rate for 48 h at room temperature. The resulting digestive solution could be neutralized using 10× PBS, NaOH (1M) and ddH2O to prepare a hDAT-pre-gel. Leave the liquid pre-gel at 37°C for about 15 min, the gelation will occur, showing a semi-solid gel.

**Staining and microstructure of hDAT-gel**

Diamidino Phenylindole (DAPI) staining was performed on native adipose tissue and hDAT-gel respectively to verify the degree of decellularization. Masson staining was used to observe changes in collagen arrangement. Scanning electron microscope (SEM) was performed to observe surface microstructure of hDAT-gel. The sample was put into the electron microscope stationary liquid (G1102, Servicebio) for 2h, and then 1% osmic acid (Ted Pella Inc) 0.1 M phosphate buffer for another 1–2h. Then the fixed sample was dehydrated using alcohol at different concentrations in sequence from 30% to 50% to 70% to 80% to 90% to 95% to 100%. After that, the dehydrated sample was put into a critical point dryer (K850, Quorum) for drying. Next, gold was sprayed for 30 s in the ion sputtering apparatus (MSP-2S, IXRF). The final sample was observed and images were collected under the scanning electron microscope (SU8100, HITACHI).

**Culture of ADSCs in hDAT-gel in vitro**

The ADSCs were mixed with liquid hDAT pre-gel at the ratio of 1×10⁶/ml. Then the pre-gel containing ADSCs was placed in an incubator at 37°C. Culture medium was added once the pre-gel got solidified. HE staining of the ADSCs-embedded hDAT-gel was performed after 2 days. Survival of cells in the gel after 2 weeks was detected by the Live/Dead™ Viability Kit (Invitrogen) and observed by laser scanning confocal microscope (PerkinElmer UltraVIEW VoX Live Cell Imaging System).

**Observation of ADSCs-embedded hDAT-gel in vivo**

A mixture of liquid hDAT pre-gel with ADSCs was prepared in vitro. Male (Nude) mice aged 8 weeks (provided by the Academy of Military Medical Sciences, Beijing, China) were injected subcutaneously using a 1 ml syringe. After about 15 min, skins of mice were cut to observe the state of ADSCs-embedded hDAT-gel in vivo.
Results

Further process of decellularized adipose tissue into hydrogel

After 5 days of decellularization, the yellow native adipose tissue (Figure 1(a)) became white mass (Figure 1(b)) with certain ductility. Then the lyophilized decellularized adipose tissue (Figure 1(c)) was cut to 1–2 mm³ granules (Figure 1(d)) and further ground to powder (Figure 1(e)). After that, the lyophilized powder was digested and dissolved by pepsin in acid environment to viscous solution (Figure 1(f)), which could still go through a 23 G syringe needle (Figure 1(g)). The neutralized digestive liquid could be physically crosslinked and self-assembled by adjusting the temperature at 37°C for about 15 min, which finally present a semi-solid gel (Figure 1(h)).

Characterization of hDAT-gel

DAPI staining showed that the decellularized adipose tissue had almost no blue-stained nuclei compared with native adipose tissue (Figure 2(a) upper). Masson staining showed significant changes in the arrangement of collagen fibers after decellularization, which no longer had cellular structures but more uniform collagenous arrangement in hDAT-gel than native adipose tissue (Figure 2(a) lower). The scanning electron microscope results of hDAT-gel also demonstrated a microstructure of uniform and staggered collagen fibers (Figure 2(b)).

Cells survived well in the hDAT-gel in vitro

After centrifugation and precipitation, the ADSCs were mixed with liquid hDAT pre-gel evenly without bubbles (Figure 3(a)). The liquid gel containing ADSCs would become semisolid when placed in an incubator at 37°C for about 15 min (Figure 3(b)). And the cell-embedded gel could still remain semisolid at 37°C at week 3 (Figure 3(c)). HE staining showed the cells were scattered in the gel on day 2 (Figure 3(d)). The 3D reconstructed image by laser confocal scanning showed that the cells can survive and proliferate well in the three-dimensional gel (Video 1). Besides, there were still a large number of green dyed living cells in the gel at week 2, and they were still evenly layered (Figure 3(e) and (f)).

ADSCs-embedded hDAT-gel gelatinized well in vivo

When the pre-gel containing ADSCs was injected subcutaneously into the nude mice, a bulge formed at the injection site immediately (Figure 4(a)). After 15 min, we observed that the hDAT pre-gel containing ADSCs formed a semi-solid gel with cell-embedded in situ at body temperature (Figure 4(b)).

Discussion

Researchers have been seeking a certain balance between complete decellularization and structural preservation about decellularization of adipose tissue. After all, this would be very important for the future safety of
decellularized adipose tissue in clinical applications. So far, Flynn’s non-detergent protocol was considered to be the most effective among all the comprehensive methods. In the practical process of decellularization, we have several points to share. First, slight mechanical stirring of adipose tissue before freezing-thawing was necessary, which would be conducive to comprehensive contact between the tissue and solvent. Second, each freezing-thawing must be done thoroughly, which would help to maximize the destruction of adipocytes. Third, keep the rotating speed of the shaker at about 100 rpm, not too high.

The principle of lyophilization was that ice crystals formed at low temperature are vacuumized out of the sample during sublimation. The more ice crystals formed, the more pores of sample were left. We could add a little water before lyophilization, so that the lyophilized tissue was more likely to be cut. The chopped granules were rapidly frozen in liquid nitrogen to increase hardness and brittleness, which was conducive to grinding. The final viscous solution digested by pepsin can be stored at −80°C and dissolved naturally at 4°C when used.

The initial PH of digestive solution was about 3.0, which could guarantee sterility. By adding salt and adjusting the PH, it was close to the physiological state. At this time, the neutralized digestive solution was called pre-gel. The hDAT pre-gel was temperature sensitive, which was liquid at 4°C and semi-solid at 37°C. As the temperature rised, the pre-gel could self-assemble for physical crosslinking. Taking advantage of these characteristics, we could mix the liquid hDAT pre-gel with ADSCs in vitro, and then delivered the mixture into a corresponding site in vivo with minimally invasive injection, providing the cells with ecological niches at body temperature (37°C).

Both DAPI and Masson staining confirmed that the degree of decellularization was complete with Flynn’s method. The composition of hDAT-gel was mainly collagen, which was more uniform after digestion. Under the

Figure 2. Characterization of hDAT-gel: (a) DAPI and Masson stainings of native adipose tissue and hDAT-gel. Scale bars represent 100 μm and (b) scanning electron microscope of hDAT-gel at different magnification. Scale bars from left to right represent 500 μm, 20 μm, and 5 μm respectively.
scanning electron microscope, the collagen fibers in the hDAT-gel were clearly arranged in staggered order, with high node density and uniform microstructure.

When mixing the cells with the gel, do not blow bubbles that would affect the formation of semi-solid gel. Cells suspended and exposed to the gel grew freely within the gel, which were different from the spindle shape in the two-dimensional environment. The 3D images reconstructed by confocal scanning showed that the cells were still layered and distributed evenly in the

Figure 3. Survival of ADSCs in the hDAT-gel in vitro: (a) the hDAT pre-gel mixed with the precipitated ADSCs, (b) the semi-solid hDAT-gel containing ADSCs after 15 min in the incubator at 37°C, (c) the ADSCs-embedded hDAT-gel at week 3, (d) HE staining of the ADSCs-embedded hDAT-gel on day 2. Black arrows indicate the ADSCs in the hDAT-gel. Scale bars represent 100 μm. (e) the 3D reconstructed image by laser confocal scanning of the ADSCs-embedded hDAT-gel viewed from the front at week 2. Green-dyed cells represent living cells and red-dyed cells represent dead cells. 1 unit represents 141.84 μm, and (f) the 3D reconstructed image by laser confocal scanning of the ADSCs-embedded hDAT-gel viewed from the side at week 2. Green-dyed cells represent living cells and red-dyed cells represent dead cells. 1 unit represents 141.84 μm.

Figure 4. Observation of the hDAT pre-gel containing ADSCs after injection subcutaneously in vivo: (a) a bulge formed at the injection site immediately and (b) the formed semi-solid gel with cell-embedded in situ at body temperature.
gel at week 2. These results indicated that ADSCs survived well in the hDAT-gel in vitro. The hDAT-gel could serve as a culture platform for ADSCs. As mentioned above, ADSC has shown great potential for clinical application in many aspects. We believe this would furnish some important practice basis for further clinical application of the injectable ADSCs-carrying hDAT-gel. In the following application research, we can consider using it as wound healing dressing containing stem cells and as an injectable scaffold material for tissue regeneration.

Conclusion

All results in this study confirmed that the hDAT pre-gel could carry ADSCs to the corresponding site, and the ADSCs-embedded hDAT-gel in-situ occurred at body temperature, providing a culture platform for human adipose-derived stem cell delivery.

Declaration of conflicting interests

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Supplemental material

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