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Exploration of the Improved Preparation Method of Rabbit Acellular Adipose Matrix

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Abstract: Tissue engineering requires biorepair materials with higher cost performance, and acellular adipose matrix has broad clinical application space. Therefore, we have improved and explored the preparation method of acellular adipose matrix of rabbit, evaluated its cytocompatibility, and verified the feasibility of its preparation method and its potential as a tissue repair material. The adipose tissue derived from rabbits was decellularized by an improved method. The appearance morphology and DAPI staining after preparation were observed, and the effects of the extracellular matrix of rabbit decellularized cells on the cytoskeleton morphology and migration ability of adipose-derived stem cells were studied. At the same time, in vivo animal transplantation experiments were carried out. The rabbit acellular adipose matrix in this experiment is gelatinous and milky white. DAPI staining leaves no residual cellular components. There are no abnormalities in the cytoskeleton or physiological activities of the cells. The prepared acellular adipose-derived extracellular matrix can be induced to form lipids in vivo. By applying the improved decellularization method, the rabbit acellular adipose matrix has no toxic effect on the cells can be successfully prepared.

1. Introduction

The acellular matrix produced by decellularization technology retains the ultrastructure and bioactive components of natural tissues and is a biomaterial with great application prospects. At present, various tissues have been studied for the preparation of extracellular matrix, including adipose tissue [1], dermis [2], trachea [3], small intestinal mucosa [4], cartilage [5], lung [6], kidney [7] and pericardium [8], etc. In the research of soft tissue repair, Acellular Adipose Matrix (AAM) has attracted much attention due to its natural structure. Inspired by the clinical application of porcine acellular dermal extracellular matrix and considering the experimental cost and economic benefits, in this experiment, we explored the preparation method of rabbit acellular adipose matrix in order to provide a more superior source of acellular fat extracellular matrix for clinical practice.

2. Method

Five healthy female New Zealand white rabbits (about 14 weeks old, weighing approximately

3kg (purchased from the Experimental Animal Center of Huadu District, Guangzhou City, license number SCXK(Yue)2019-0023) were selected. Among them, 3 rabbits were used for AAM preparation and 2 rabbits for AAM transplantation. All received intramuscular anesthesia with the new methamphetamine. All operations are carried out under aseptic conditions and are prophylaxis with antibiotics.

2.1. Preparation of rabbit acellular adipose matrix

After successful anesthesia of the animal, subcutaneous fat is taken from the groin area. Considering the characteristics of rabbit fat, we improved Flynn's classic method [1] in order to achieve a better decellularization effect. The specific preparation process is as follows: After thorough cleaning, use scissors to remove as much of the fascia and blood vessels as possible. Then put it into a tissue homogenizer and crush it (2000 RPM for 10 minutes), and then freeze and thaw it repeatedly. Transfer the sample into trypsin for digestion. Then add 99.9% isopropyl alcohol for 24 hours. After the grease was removed, the samples were washed with buffer solution and then digested with trypsin for 6 hours again. The digested samples were subjected to nucleic acid removal treatment for 16 hours. Finally, carry out the second degreasing treatment after 6 hours. All the successfully prepared AAM were sterilized (irradiated with 60Coγ) and stored in a -80℃ refrigerator for future use.

2.2. DAPI staining

Paraffin sections were prepared from rabbit adipose tissue and rabbit AAM. Rabbit adipose tissue and rabbit AAM sections were stained with DAPI staining solution. The nuclear staining of rabbit adipose tissue and rabbit AAM sections was observed by fluorescence using an inverted phase contrast fluorescence microscope.

2.3. Isolation and culture of rabbit Adipose-derived stem cells (ADSCs)

Take the fat tissue from the groin area of the rabbit, remove the fascia and blood vessels, and cut it into small pieces as much as possible. Add an equal volume of 0.1% type I collagenase and shake at 37 ℃ for digestion, then centrifuge for 5 minutes. Remove the supernatant, resuspend it, and then add red blood cell lysis buffer for washing and filtration. Add the freshly isolated cells to the T25 culture flask at a density of 5×106 cells /100 mm. Change the culture medium every three days. Cells can be used for plate laying after being cultured to the third generation.

2.4. The influence of acellular adipose matrix of rabbit on the cytoskeleton

Third-generation rabbit adipose-derived stem cells were used to lay the plates. In the experimental group, rabbit AAM was placed in the culture chamber and culture medium was added until the AAM was fully submerged. In the control group, only the same amount of culture medium was added. The culture chambers were taken out at 24 hours and 72 hours respectively. After PBS rinsing, 4% paraformaldehyde was added to fix the cells. Add 0.1% Triton X-100 solution for 5 minutes. Add the cytoskeletal fluorescent staining agent Ficyclin (FITC-labeled) for 20 minutes, rinse, and then add the nuclear fluorescent staining agent DAPI for 3 minutes. This process requires attention to avoiding light. Take pictures with an inverted fluorescence microscope.

2.5. The influence of rabbit acellular adipose matrix on the migration of adipose-derived stem cells

Soak the rabbit AAM in the culture medium for 24 hours and then take out the soaking solution. The third-generation rabbit ADSCs were inoculated into 6-well plates in two groups. The experimental group was added with AAM soaking medium, while the control group was added with ordinary medium. After the cells have fused to 100%, gently scrape along the marked line with the tip of a 1-milliliter pipette, trying to keep the width of the scratch in each well consistent. Rinse with PBS. The cell migration was observed under an inverted microscope at 0 hours, 12 hours, 24 hours and 48 hours respectively, and the quantitative comparison of cell migration rates was conducted using Image J.

2.6. Study on adipogenic differentiation of rabbit acellular adipose matrix in vivo

After successful anesthesia of the animal, a total of six areas are divided with the midline of the posterior spine on the back as the dividing line. Subcutaneous transplantation and injection of rabbit AAM were performed in the planned area, with 0.2ml injected at each injection point. The specimens were removed three months after AAM injection. They were dehydrated with gradient ethanol and then embedded in paraffin. They were continuously sectioned to a thickness of approximately 5μm. Immunohistochemical staining of perilipin was performed on the sections.

2.7. Data analysis

The analysis was conducted using SPSS statistical software. Data were expressed as mean ± standard deviation, and paired t-tests were used for comparison between the two groups. The test level $\alpha=0.05$.

3. Results

3.1. The rabbit acellular adipose matrix was in a gel-like state

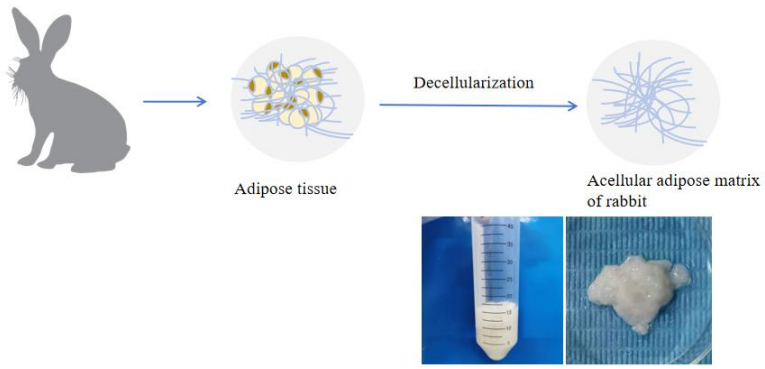

Figure 1. The preparation process of acellular adipose matrix, and the successfully prepared tissue presents a milky white jelly-like state

Referring to the commonly used areas for liposuction in the human body, in order to reduce the technical difficulty, the fat collection site is the groin. After undergoing our decellularization process that combines mechanical treatment with chemical and biological actions, rabbit adipose tissue appears as a gel-like substance, is white in color, and has certain toughness and ductility. Compared with adipose tissue, the texture is more resilient after decellularization treatment, which

is considered to be related to the remaining extracellular matrix after the removal of adipocytes. The volume after successful preparation was significantly reduced compared with before (Figure 1).

3.2. The rabbit acellular adipose matrix is completely decellularized

The prepared rabbit AAM was subjected to immunofluorescence DAPI staining. Under an inverted fluorescence microscope, no obvious blue-stained nuclear structure was observed. However, in untreated rabbit adipose tissue, DAPI staining showed a large amount of blue fluorescent nuclear staining (Figure 2). This phenomenon indicates that the rabbit AAM has been thoroughly decellularized after treatment.

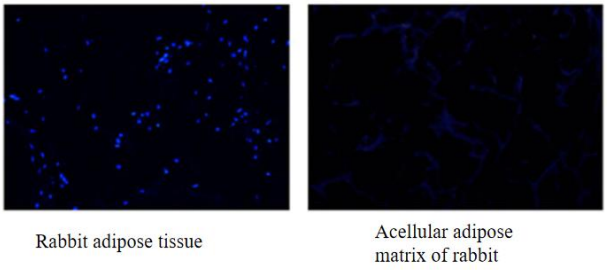

Figure 2. DAPI staining of rabbit adipose tissue and acellular adipose tissue

3.3. During co-culture, the morphology of adipose-derived stem cells was normal spindle-shaped, and the cytoskeletal immunity was normal

In the co-culture experimental group of rabbit AAM and ADSCs, it could be observed that the 24-hour cytoskeletal immunofluorescence staining showed green fluorescent-labeled normal cytoskeletal microfilament structures, which were not significantly different from the cytoskeletons in ordinary culture dishes. The nuclei were relatively large, and most of the cells were spindle-shaped or polygonal (Figure 3).

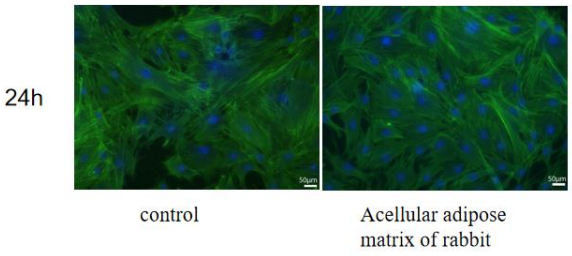

Figure 3. Immunofluorescence staining of rabbit ADSCs cytoskeleton in the control group and the experimental group (200X)

3.4. The rabbit acellular adipose matrix does not have a negative impact on the migration of rabbit adipose-derived stem cells

In the scratch test (Figure 4), both groups of rabbit ADSCs treated with AAM immersion solution and common medium achieved 100% fusion within 48 hours. At 12 hours and 24 hours, although it was observed that the migration of the rabbit AAM immersion culture group was faster than that of the control group, after statistical analysis, there was no statistically significant difference in the cell migration rate between the two groups, that is, the rabbit AAM treated with

multiple reagents did not have a negative impact on the cell migration ability.

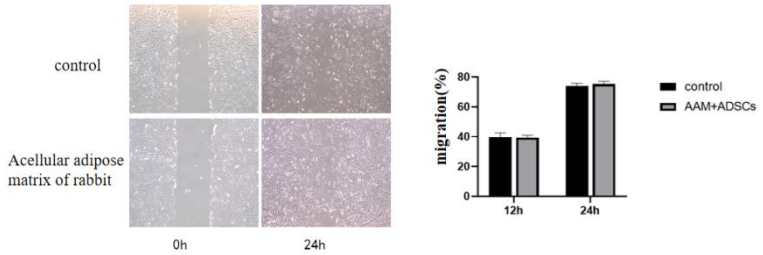

Figure 4. Cell scratch experiments and statistics of co-cultured rabbit AAM, common medium and rabbit ADSCs

3.5. Adipogenesis can be observed in vivo transplantation of the rabbit acellular adipose matrix

After in vivo transplantation of rabbit AAM, at the third month, we took out the specimens and performed immunohistochemical staining of perilipodroplet proteins: the formation of brown-positive enveloped round adipocytes could be observed (Figure 5). It indicates the regeneration of new adipose tissue.

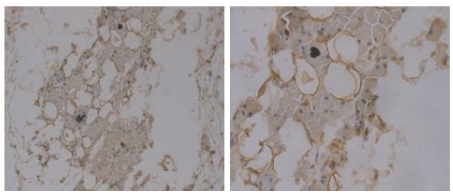

Figure 5. Acellular adipose matrix of rabbit was injected in vivo for three months

4. Discussion

With the development of tissue engineering, acellular adipose extracellular matrix has been deeply explored due to its wide availability and excellent potential in tissue regeneration [9]. In addition to having great prospects in the field of adipose tissue regeneration, the modified acellular adipose matrix can also be applied in other tissue engineering fields, including cartilage and bone tissue engineering, neural tissue engineering and skin tissue engineering [10].

During the preparation of acellular adipose matrix, we carried out a series of cell removal and nucleic acid removal treatments, so the obtained acellular tissue was a low immunogenic material derived from the organism itself [11]. It is precisely because of the low immunogenicity of the acellular matrix that the use of xenogeneic species becomes possible. Rabbits were chosen as the experimental subjects because, as mammals, they have low breeding costs, strong reproductive capabilities, short cycles, fast growth rates, and can obtain a large number of samples in a short period of time, with high economic benefits. If the clinical application of rabbit AAM is realized, it can provide a more cost-effective biological material option for clinical practice.

At present, the main methods for decellularization preparation include mechanical treatment, chemical permeation, and the use of biological enzymes, etc. However, the use of biological enzymes is expensive and time-consuming. If not well controlled, it may also affect the yield and quality of AAM. The residues of chemical reagents are prone to cause cytotoxicity, so scientists are constantly improving their methods. Liu et al. [12] and Tang et al. [13] found that by increasing the

intensity of mechanical destruction, the cellular components in adipose tissue could be effectively removed. Yang et al. [14] successfully achieved decellularization and decellularization of AAM by applying mechanical treatment and hypertonic buffer flushing, providing a non-chemical and enzyme-free preparation method. Qi[15] et al. replaced the enzyme treatment step in the classic FLYNN with mechanical treatment, reducing costs and the risks of heterologous antigens and pathogens. Feng et al. [16] compared three mechanical methods for enzyme-free preparation of AAM, namely ultrasonic stirring, homogenization, and cryogenic grinding, and found that ultrasonic stirring treatment had the best effect in preparing AAM.

All these methods share a common feature: when preparing decellularized tissues, mechanical processing is used to extract the tissues, achieving the lowest cost and lowest toxicity to remove the cells in the tissues to the greatest extent possible. At the same time, it is necessary to avoid damaging the original components during the decellularization process to preserve the relatively complete natural extracellular matrix structure and provide a physiological basis for the good application of the scaffold.

At present, the most commonly used mechanical treatment methods are ultrasonic treatment, homogenization treatment and cryogenic grinding. Cryogenic grinding uses grinding balls as the medium to break the sample through impact, compression and friction. However, this method is not suitable for grinding adipose tissue. Research has found that many adipocytes have not been damaged at both the macroscopic and microscopic levels, and there is also a risk of increasing the mixture of other impurities [17]. Ultrasonic treatment converts electrical energy into acoustic energy and causes cell rupture through acoustic cavitation [18,19]. However, under high frequency and high voltage, this treatment generates a large amount of heat [20], which can easily lead to thermal denaturation of active proteins, affecting the lipidization of AAM. Moreover, the ultrasonic treatment process is more complex and costly. Homogenization treatment, on the other hand, employs a high-speed shear dispersion method, generating strong liquid shear force and high-frequency mechanical action, which can effectively shear, tear and mix tissues [21]. The homogenizer has a good crushing effect on large-particle high-viscosity solid-liquid mixtures and is suitable for the crushing of adipose tissue [22,23].

After the preparation was completed, we conducted DAPI staining detection on the prepared samples, confirming that our experimental decellularization was complete and this method was feasible for preparing acellular matrix of the rabbit . However, various chemical reagents were used in the preparation of acellular adipose extracellular matrix. Although we shortened the action time of the chemical reagents by adding mechanical treatment and also carried out multiple neutralization and washing, whether the prepared rabbit AAM had a negative impact on the cells still needs further verification.

Therefore, we conducted cell experiments using the successfully prepared rabbit AAM. From the scratch experiment, it can be concluded that the healing rate of adipose-derived stem cells in the rabbit AAM group was higher than that in the culture medium control group, but the difference was not statistically significant. This proves that the rabbit AAM we prepared has no negative impact or toxic effect on the normal physiological activities of cells. However, the healing rate was not statistically significant, which was considered to be related to the fact that the growth environment factors in the in vitro experiment were single and did not have the influence of complex interactions within the body. If conditions permit, in vivo experiments can be added in the future to further verify this phenomenon. In addition, we also performed cytoskeletal staining on adipose-derived stem cells co-cultured with rabbit AAM, and the cytoskeleton was no different from that of adipose-derived stem cells cultured in ordinary medium. This further indicates that the rabbit AAM we prepared has no toxic effect on the cells, does not affect the normal morphology and physiological functions of the cells, and has good tissue compatibility. Finally, we further confirmed

in in vivo transplantation experiments that the acellular adipose matrix prepared by this method has the ability to induce fat regeneration.

For this study, we prepared the acellular adipose matrix of rabbit using an improved traditional method. In subsequent research, we can continue to adopt the improved methods from various studies, compare the differences in the preparation of acellular adipose matrix of rabbit using different methods, and then find the optimal preparation plan to lay the foundation for the clinical application and commercialization of acellular adipose matrix of rabbit t in the future.

5. Conclusions

In summary, we prepared acellular adipose matrix derived from rabbit by using the modified classical FLYN preparation method and verified that it has no toxic effect or negative impact on cells. It provides a new idea for soft tissue repair engineering in clinical practice.

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