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Assessment of Sheep Omentum for Soft-tissue Engineering Scaffolds

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Abstract

IN THIS STUDY, three-dimensional bio-tissue scaffolds were prepared in vitro, which play an important role in tissue engineering. The aim of this research was to study the use of sheep omentum in the manufacture of soft tissue engineering scaffolds used in the laboratory, as this biosynthesis is characterized by its availability and cheap price. The biological metabolism of scaffolds also plays an important role in the process of new tissue formation. A porous biomaterial scaffold was designed using connective tissue from sheep omentum, which was successfully prepared by a freeze-drying method that included a decellularization process and the process of removing cells was confirmed using DAPI staining. The following properties of the scaffold were then measured, such as the biological properties of the resulting tissue, as well as Glycosaminoglycans (GAG) and metabolic activity (MTT) tests which proved that this tissue formed was not toxic to the cells seeded on it , structural properties, mechanical tests, and microscopy . Scanning electron imaging (SEM).

Keywords: Tissue engineering of soft tissues; Design and fabrication of sheep omentum scaffold; Biodegradable and bioresorbable natural material

Introduction

Tissue engineering and stem cells are interdisciplinary fields that use materials and life sciences to create regenerative medicine. It helps solve tissue damage caused by disease or trauma, and develops biological substitutes to restore, maintain, or improve function in failing organs [1-3].

Engineering methods for skeletal muscle tissue regeneration hold promise for treating muscle diseases like muscle dystrophy, traumatic injury, and aggressive tumor ablation. Degradable biomaterials induce new tissue and cell ingrowth, or serve as temporary scaffolds [4-6]. The study uses patient's cells or immunologically inactive allogenic or xenogenic cells to engineer tissue scaffolds, potentially replacing lost tissue function and offering new therapeutic options for skin damage in burns. Tissue engineering involves cells and culturing methodologies, crucial for designing improved combinations. Recognizing tissue characteristics helps design improved techniques, potentially aiding in pediatric patient management [7-11].

Current studies focus on creating safe, reliable, economic, and physiologically acceptable devices using biomaterials for body repair [12]. This tension-free repair reduces post-operative pain, recovery time, and repetitions, gaining recognition in the medical field [13]. Researchers are increasingly using biomaterial scaffolds for reconstruction, repairing traumatic wounds from war, traffic accidents, and natural disasters. These cheap, ideal biomaterials, such as ceramic, polymeric composites, and biologics like heart valves and skin, are being researched [14,15]. Biomaterials for skin burn repair should have sufficient strength, no hypersensitivity reactions, and biocompatibility for tissue ingrowth, ensuring long-term mechanical strength maintenance [16,17]. The greater omentum is utilized for clinical applications, with grafting for surgical purposes available in literature [18]. Adipose tissue and connective tissue rich sheets can be obtained from sheep or goat abdominal cavities. Saline, derived from naturally derived materials, preserves the omentum, which is used in various surgical cases, including chest surgery, chronic empyema, and lung transplantation to prevent bronchial dehiscence

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[19,20]. Tissue muscle recovery may fail due to lack of strength over time, affecting clinical applications. Adequate tensile properties are needed for omentum tissue. For the understanding of the biological response and mechanical properties of degradable biomaterials is crucial. Therefore, the seeding with myoblast cells on the scaffold before the implantation improves properties, requiring in-vitro and in-vivo evaluation.

Material and Methods

Preparation natural biodegradable scaffold

A. Sheep omentum

Great omentums from healthy adult sheep were collected and inspected for infection before being removed, packaged in cold saline, and transported to the University of Baghdad.

B. Scaffold decellularized and freeze drying

The great omentums were cleaned, trimmed, washed, rinsed, transferred to a sterile bottle containing sodium hypochlorite, washed, and stored overnight at 4°C. The freeze-thaw cycle is a decellularization technique used to create scaffolds for organ repair, offering advantages over synthetic compounds, including preserved natural microenvironment features, and is used in evaluating omentum decellularization. The freeze-thaw cycle was utilized to remove cells from tissue, disrupting cell membranes and causing cell lysis through intracellular crystal formation and binding to DNA strands [21]. The great omentum sheets were freeze-dried using a freeze-drier, disinfected with 70% alcohol, and maintained at -40°C. After reaching room temperature, samples were packed in airtight polyethylene bags, sealed, and labeled. Stored at room temperature, samples were sterilized by gamma irradiation, which was done the gamma irradiation at Tabriz University of Medical Science, following the TLNT (Tabriz Laboratory for Nuclear Science and Technology)[22].

Histological methods were used to evaluate the surface appearance of the extracellular matrix (ECM) components and basement membrane after decellularization. General morphology was examined by staining scaffold histological slices and intact tissue with hematoxylin and eosin (H&E). In short, the tissue specimens were fixed with 10% formalin. Higher graded concentrations of xylene and ethanol were then used to carry out the dehydration process. After paraffin fixation, transverse slices 5 µm thick were cut from the samples. Tissue slices were deparaffinized in xylene and rehydrated using ethanol concentrations that were gradually lowered. The slices were stained with H&E in accordance with standard procedures, and the resulting slides were inspected using a light microscope (Olympus, Japan) [23].

C. Characterization of natural biodegradable polymer in-vitro.

DAPI Staining

4'-6-diamidino-2-phenylindole (DAPI) dye, a blue fluorescent dye to nucleic acids, was used as a marker to reveal the cell nucleus, this coloration was applied to scaffolds in this project to ensure decellularization. After deparaffinization and dehydration, slides were placed in PBS for 5 minutes, a drop of diluted dye solution was poured on the sections and after 10-20 A minute slide was placed on the samples and then diluted with a dye solution. Lastly, the sections were examined under a fluorescent microscope and photographed. Therefore, there were many points for evaluated of decellularized the tissue which included the cell removal efficiency, tissue ultrastructure preservation, toxicity, biocompatibility, biodegradability, and mechanical resistance in order to enhance the efficacy of decellularization methods. [24-27].

GAGs

The amount of GAG in decellularized scaffold and control groups was determined by lysing 15 mg scaffold and control samples in an enzyme solution (Papain enzyme) and incubating them for 16 hours at 65 degrees. The samples were centrifuged at 4 degrees for 15 minutes at 6000 g, then 200 µL of GAG reagent was applied to each well in a 96-well plate, incubated at room temperature for 30-60 seconds, and its light absorbance was measured between 510 and 560 nm [22].

Mechanical tensile test

A universal testing machine (SANTAM-STM20, Tehran, Iran) was used for uniaxial tensile tests on all samples, including the control (intact tissue), at room temperature. PBS was used to keep the scaffold and control moist. Soft-tissue clamps were used to mount each specimen on a uniaxial tensile testing apparatus. then, each sample was stretched with the 1 mm/s strain rate until it becomes torn. From each group 5 samples were evaluated [40].

MTT test assay

The MTT assay was used to examine the growth and multiplication of cells on scaffolds [24]. The enzyme succinate dehydrogenase transforms water-soluble tetrazolium salt into an insoluble purple formazan, which can be measured using a spectrophotometric approach at 570 nm wavelength. The first step involves culture cells on scaffolds in 96-well culture flasks. Cells were cultured on a scaffold, then 20µl of MTT solution was added to

each well under sterile conditions for 24, 48, and 168 hours. After a 4-hour incubation period, 200 µl of DMSO was added to each well to dissolve the purple formazan crystals. Once homogeneous solutions were produced, the optical absorption of each well was measured using an ELISA reader. The process was repeated for each well. Following scaffold culture [41].

Scanning Electron Microscopic Analysis of the Scaffolds

Scanning electron microscopy was used to evaluate the morphology of the scaffold derived from sheep omentum. The SEM evaluation was conducted using Electron Microscopy Unit, Centre of nano-technology and advance materials / University of Technology for SEM analysis (Tuscan Vega 3rd Generation-England) at accelerating voltage of 20-30kv. For this purpose, both control and scaffold samples were fixed in 2.5% glutaraldehyde. Then, samples were dehydrated by being placed in different percentages of alcohol. Finally, the samples were examined using SEM microscopy after being dried and coated by a gold-palladium layer [28].

Results

Histological evaluation:

The H&E staining findings showed that decellularization was complete, indicating the removal of lipids and cell nuclei (Fig.1, A and B). Furthermore, the dark blue staining makes the nuclei of the control group (intact tissue) evident in the corner of the cell (black arrow) (Fig.1, C and D).

DAPI Staining

The bright blue specks in the photograph represent the nuclei of the cells. In this work, DAPI labelling was used to compare the decellularized tissue to normal tissue in order to assess the absence of nuclei. The results show that all of the cells in the scaffold sample have been completely eliminated as compared to the control sample. As can be seen in the photograph, the decellularized scaffold sample consists solely of extracellular matrix devoid of any cells.

GAG estimate

The glycosaminoglycan test findings showed that there was less GAG recovered from the scaffold samples than from the control samples. It may be stated that the decellularization process has preserved the scaffold sample's nearly high level of GAG, Almost the same as in raw tissue The scaffold sample has 5.15 µg/mg of GAG, whereas the control sample has 6.24 µg/mg, as shown in Fig. 3.

Mechanical tensile test

The greatest tensile force (Stress) applied by scaffolds was 1.78± 0.6 N, which was decreased significantly from 2.89± 0.4 N ($p = 0.0003$) for the control (intact tissue) (**Fig. 4**). At maximal force, the scaffold's elongation (strain) was 2.95 mm, whereas the control's (intact tissue) was 8.07 mm. This difference was statistically significant ($p = 0.0001$).

MTT test assay

The MTT assay revealed that there were no appreciable differences in cell viability or proliferation at 24, 48, or 168 hours between the scaffold-seeded cells and the control group of cells grown in 96-well plates. These findings showed that the scaffold did not harm the implanted cells. data are shown as the ratio of the mean of the optical density of the treated groups to that of the control group (Fig. 5). The scaffold-seeded cells and the control group of cells cultured in 96-well plates did not significantly vary in terms of cell viability or proliferation after 24, 48, or 168 hours after scaffold culture, according to the results of the MTT experiment. These results demonstrated that the transplanted cells were not harmed by the scaffold, on the contrary, the scaffold enhanced the growth and proliferation of cells and increased the density measured by the ELISA device.

The data is presented as the mean optical density of the treatment groups divided by the mean optical density of the control group (27,28,29).

Scanning Electron Microscopic Analysis of the Scaffolds

The SEM micrographs illustrate the scaffold's ultrastructure was maintained, all of its lipids were removed, and elastic fibers and collagen, two types of extracellular matrix, were visible (Fig 6, A). Additionally, the intact tissue's (control) SEM micrograph revealed that it contains a high concentration of lipids, which are depicted as droplets in the SEM pictures (Fig 6, B).

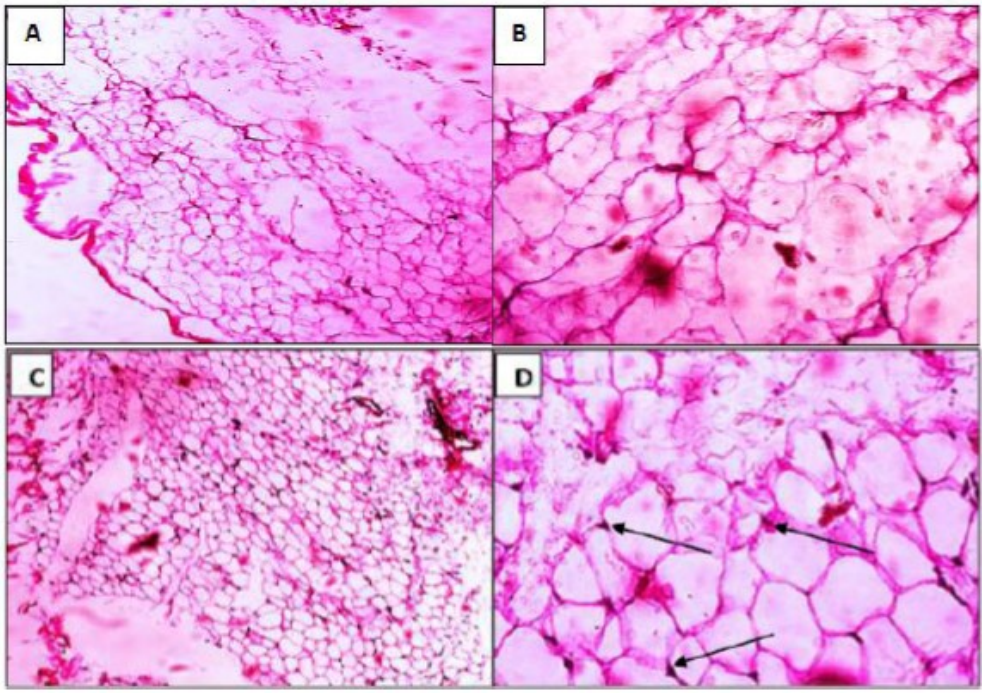

Fig. 1. A and B scaffold tissue, C and D control tissue

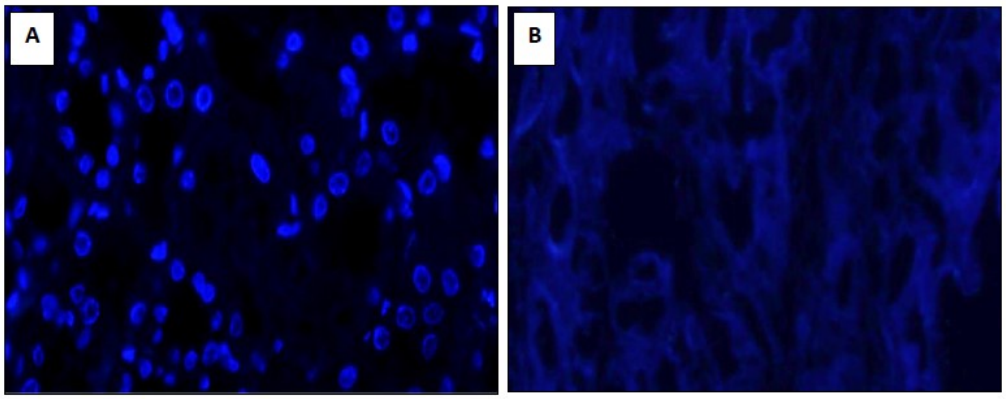

Fig. 2. A) Control group (Intact tissue), B) Scaffold (decellurized matrix)

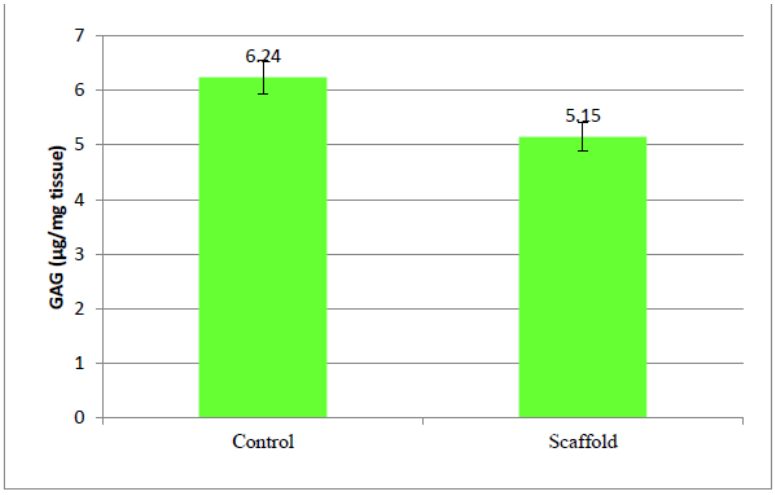

Fig. 3. Showed the differed GAGs content between control and scaffold samples.

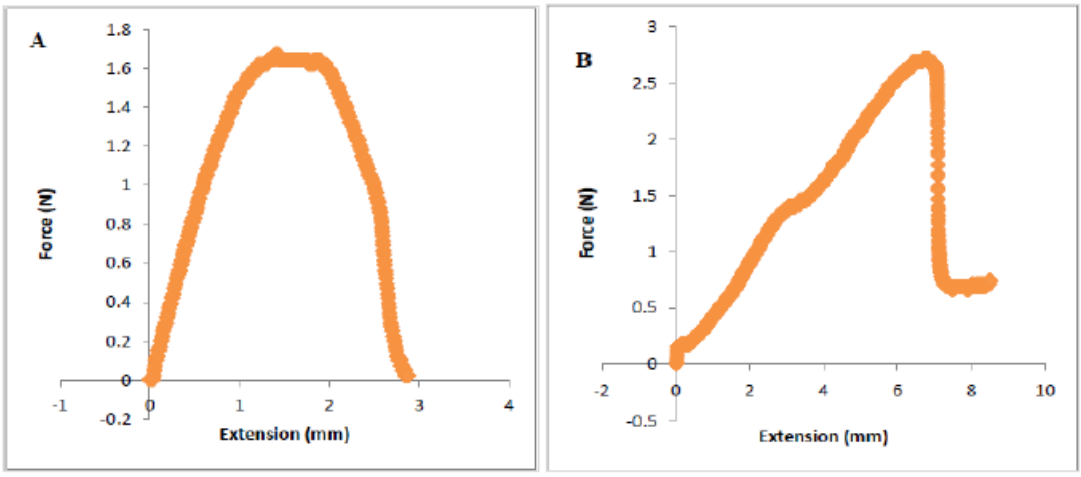

Fig. 4. Stress-strain curve of scaffold (A) and control (Intact tissue) (B)

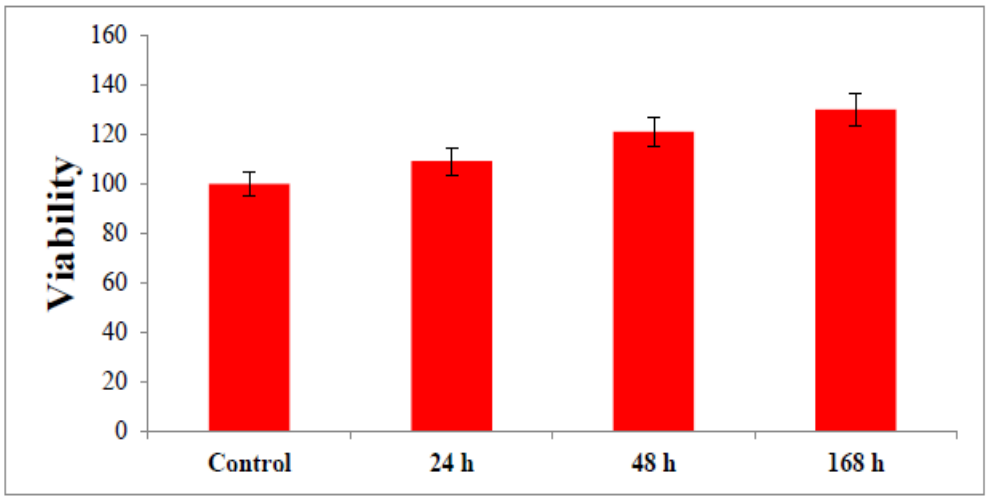

Fig. 5. Showed the evaluation of the scaffold's biocompatibility. The viability and proliferation of cells seeded onto scaffold was compared using MTT assay with cells cultured in 96-well plates as control. The experiments were performed in triplicate.

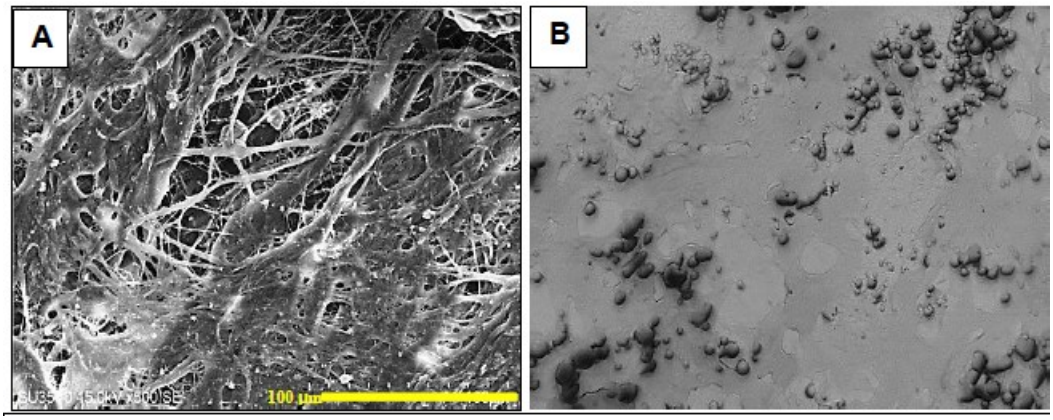

Fig. 6. Ultra structure micrographs (A) Scaffold after lipid removed (B) control before the lipid removed

Discussion

The result of Histological evaluation by (H&E staining and DAPI staining) confirms the removal of cells and DNA fragments from the matrix to prevent toxicity and any immune response after that in the subsequent steps of cell culture on the scaffold , this is consistent with the results of [30].

The results of the determination of GAGs showed that they were preserved in an excellent percentage and there was no relative difference between them and those found in the raw tissue, which better enhances the adhesion of cells to the scaffold, as well as regulating the process of cell growth and stimulating cell division and multiplication, and this is corresponds with what was proven [31].

From the above, we find that the tensile strength of the scaffold produced was within the limits required for its production and adoption as a biological scaffold that can be implanted in the living body, which was proven in confirmation of what was found by [32].

In this research, it was confirmed that the manufactured bio scaffold was non-toxic to the cells. Testing using the MTT technique demonstrated that the scaffold enhanced the growth and proliferation of cells over time, which confirms the non-toxicity of the scaffold to the cells grown on it even after 168 hours, which was confirmed [33-36].

As for examination with a scanning electron microscope (SEM), it showed the fatty tissue before and after extraction fat and decellularization , and what are the shapes and diameters of the fibers present within this manufactured scaffold , as well as the diameters of the interstitial holes within this scaffold, which have a major role in the growth and reproduction of cells , as the diameters of fibers and holes have a direct effect on cell adhesion, proliferation and migration within the manufactured scaffold, positively if they are within the appropriate measurements and as mentioned [37, 38].

Conclusions

We conclude from this research that it is possible to manufacture biological scaffolds used in the process of grafting lost organs and structures from tissues taken from other living organisms without any immunological conflict, which opens the way to saving many lives.

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Conflicts of interest

There are no conflicts of interest declared by the authors.

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تقييم ثرب الأغنام للسقالات الهندسية ذات الأنسجة الرخوة

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في هذه الدراسة، تم تحضير سقالات الأنسجة الحيوية ثلاثية الأبعاد في المختبر، والتي تلعب دورا هاما في هندسة الأنسجة. هدف هذا البحث إلى دراسة استخدام ثرب الأغنام في صناعة سقالات هندسة الأنسجة الرخوة المستخدمة في المختبر، حيث يتميز هذا التخليق الحيوي بتوفره ورخيص الثمن. يلعب التمثيل الغذائي البيولوجي للسقالات أيضًا دورًا مهمًا في عملية تكوين الأنسجة الجديدة. تم تصميم سقالة مادة حيوية مسامية باستخدام النسيج الضام من ثرب الأغنام، والذي تم إعداده بنجاح بواسطة طريقة التجفيف بالتجميد التي تضمنت عملية إزالة الخلايا وتم تأكيد عملية إزالة الخلايا باستخدام تلطيخ DAPI. تم بعد ذلك قياس الخواص التالية للسقالة، مثل الخواص البيولوجية للنسيج الناتج، وكذلك اختبارات الجليكوزامينو جلايكان (GAG) والنشاط الأيضي (MTT) التي أثبتت أن هذا النسيج المتكون لم يكن سامًا للخلايا المزروعة عليه، وكذلك تم اختبار الخواص الهيكلية، الاختبارات الميكانيكية، والفحص المجهري. مسح التصوير الإلكتروني (SEM).

الكلمات الدالة: هندسة الأنسجة من الأنسجة الرخوة، تصميم وتصنيع سقالة ثرب الأغنام، مادة طبيعية قابلة للتحلل الحيوي وقابلة للامتصاص.