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In Vitro Differentiation of Myoblast Cells on Sheep Omentum Scaffold to Mature Myofibers

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Abstract

The study aimed to engineer skeletal muscle tissue by used *in vitro* myoblast seeding on scaffolds to construct skeletal muscle tissue. Consequently, a scaffold made of sheep omentum and myoblast cells from rabbits was created for the healing of bodily tissues. Decellularization, mechanical testing, scanning electron microscopy (SEM) were used to analyze the structural features, and exposure to 25/Kg gamma radiation for sterilization. A seven-day-old male rabbit's thigh muscle was successfully used to extract myoblasts, which were then subjected to a purity test utilizing flow cytometric analysis. After being seeded onto a scaffold, myoblasts were cultured *in vitro* for five days. This naturally derived collagen-based scaffold showed tremendous potential for *in vitro* skeletal muscle cultivation, which can be used as a substrate for filling wound beds or delivering cells. The *in vitro* finding of myoblast-seeded omentum scaffold suggested that myoblast harvested from primary culture are able to form myotube on scaffold.

Keyword: Myoblast, Myofiber, Scaffold, Decellularization

Introduction

The science of growing live tissue to replace, repair, or enhance damaged tissue is generally referred to as "tissue engineering." Therefore, regardless of the approach, the engineered tissue may be formed *in vitro* and then implanted into the patient, or it may be created wholly *in vivo*. Three elements are necessary for tissue engineering: a growth factor that includes stimulation (induction), generation of responding cells (production), and a scaffold made of biomaterials to facilitate the synthesis of tissue, Bronzino (2006), Skalak and Fox(1988), Thomson et al.,(1995),Essa et al.,(2020) , AL-Ameri and AL-Timmemi (2018). The need for biomaterial scaffolds that can be used for reconstruction, surgery, and the repair of traumatic wounds sustained during war, traffic accidents, and natural disasters is growing, and the primary goal of most researchers is to find affordable and ideal biomaterials for the restoration of diseased tissue or organ functions. The biomaterials can be biologic (bio-prosthesis) like skin, heart valves, and other tissue grafts, or synthetic (prosthesis) like ceramic or polymeric composite. For skin burn healing or any other tissue injury, the optimal biomaterials should be strong enough, not cause hypersensitive reactions, and biocompatible enough to promote tissue ingrowth and long-term mechanical strength maintenance (Black(1992), Lai (2003), Bahaa and Khayreia (2017) , Jenan (2018) , AL-Qaisy et al(2014), Dhurgham and Al-Timmemi(2023). The first people to successfully implant in vitro cultured myoblasts into a non-muscular environment were Wering et al(2000),Sexena(1999). Dhuha (2022), Sexena (2001), Sexena (1999), Al-Mutheffer et al(2023), Hayder and Ahameed (2022) These researchers notes that in the field of stem cells and tissue engineering, myoblast transplantation has been primarily performed by injecting myoblast cell suspensions into mature skeletal muscle. The majority of the results have shown the single cells were to fuse with the host myofiber, Therefore the team grew skeletal muscle cells on a polyglycolic acid (PGA) mesh Dhuha (2022), Sexena (2001). In

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vitro, myoblasts have also been successfully seeded onto porous polymers containing polyglycolic acid, resulting in the creation of vascularized new skeletal muscle Sexena (1999), Al-Mutheffer et al(2023). According to Khayreia et al(2023), Saxena and Willital (2000), Al-Timmemi (2011), the larger omentum is now utilized as a source of natural biomaterials for a variety of therapeutic applications. Nonetheless, a wealth of clinical data on omentum grafting for surgical purposes is already accessible in the literature; however, mature sheep or goat abdomens can provide an adipose tissue connective tissue rich sheet of omentum up to 10X9 cm or greater. Materials obtained naturally, such as salt, kept the omentum intact. Omentum is commonly employed in a variety of surgical settings, such as chest surgery, chronic empyema, and chest wall reconstruction (Thomas (2020), Valerio (2019) , Helal and Hussein (2022). It is also frequently used to seal a leaky bronchus stump following a pneumonectomy. Additionally, omentoplasty can be used widely to avoid bronchial dehiscence after lung transplantation Bahaa and Khayreia (2017), Dhuha (2022). In any case, the omentum tissue needs to have sufficient tensile characteristics since over time; the muscle's capacity to heal may fail owing to a lack of strength, which might be problematic for clinical use. Understanding the biological reaction to biodegradable biomaterials as well as the anticipated mechanical characteristics of implants and tissue replacement over time is crucial. Before being authorized for use in human trials, these novel fat-derived tissue biomaterials must first be examined in vitro and then in animal models (in vivo) to enhance their morphological and biomechanical qualities

Materials and Methods

1 .Scaffold decellularized and freeze drying

Omentum decellularization is evaluated using the freeze-thaw cycle, a decellularization method that offers advantages over synthetic chemicals, such as retained natural microenvironment properties, and is utilized to generate scaffolds for organ repair. Cells were separated from tissue using the freeze-thaw cycle, which broke down cell membranes and led to cell lysis by binding to DNA strands and intracellular crystal formation Soffer-Tsur (2014). The enormous omentum sheets were kept at -40°C, cleaned with 70% alcohol, and freeze-dried using a freeze-drier. Samples were brought to room temperature, sealed, tagged, and placed into airtight polyethylene bags. The samples were kept in room temperature storage and then were subjected to gamma irradiation at Tabriz University of Medical Science in accordance with TLNT (Tabriz Laboratory for Nuclear Science and Technology) protocols for sterilization Fazelian- Dehkordi (2022). The tissue specimens were fixed with 10% formalin, dehydrated with xylene and ethanol, and then cut into 5 µm thick slices. The slices were stained with H&E and inspected using a light microscop, Suvarna et al (2012).

2 .Mechanical tensile test

In this test, all materials are using a universal testing apparatus (SANTAM-STM20, Tehran, Iran), uniaxial tensile tests were performed on all materials, including the control (intact tissue), at room temperature. PBS was used to control and preserve moisture in the scaffold. Soft-tissue clamps were used to attach each specimen on a uniaxial tensile testing apparatus. A strain rate of one millimeter per second was applied to five samples, one from each group, until the sample broke, Perez- Puyana et al(2021)

3 .Scanning Electron Microscopic Analysis of the Scaffolds

SEM was used to do a morphological examination of the sheep omentum scaffold. Using the Electron Microscopy Unit at the Centre of Nano-technology and Advance Materials / University of Technology, the SEM investigation (Tuscan Vega 3rd Generation, England) was carried out at an accelerating voltage of 20–30 kv. For this aim, 2.5% glutaraldehyde was used to fix the scaffold and control samples. After that, samples were immersed in various alcohol concentrations to remove moisture. The samples were examined under a SEM microscope following their drying and gold-palladium coating, Kashi (2014).

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4 .Myoblast Isolation and Culture

According to Witt et al (2017) the skeletal muscle from a seven-day-old rabbit's thigh muscle was extracted using a sterile surgical knife and scissors after cleaning with 70% alcohol. The skin was removed using forceps, and the muscle tissue was sliced into slurry for culture, with PBS added to maintain moisture.

The culture dish was filled with collagenase/dispase/CaCh solution for every gram of tissue, and the mincing process was left for two minutes. The mixture was incubated at 37°C for 20 minutes until it thickened into slurry. Throughout the incubation, clumps were broken apart with a plastic pipette, and large tissue fragments were removed by filtering through an 80 um nylon mesh in a sterile funnel. The cells were centrifuged for 10 minutes at 1000 rpm, resuspended in F-10-based primary myoblast growth medium, and plated in 75 cm2 tissue culture flasks. The medium was changed every two days and incubated at 37°C with 5% CO2.

The cells were extracted from a dish using PBS without trypsin or EDTA, aspirated off, and rinsed with PBS. They were preplated for 15 minutes on tissue culture flasks, and then moved to new flasks. Repeated steps were taken until most fibroblasts were removed. A small amount of PBS was left in the dish, and the dish was firmly damaged it from a sideways against a table top to dislodge the cells .

The study used a modified cell culture technique, adapted from , Springer(1997), Pavlath(1996) to harvest pure myoblasts in just three weeks.The medium was switched to F-10/DMEM-based primary myoblast growth medium, and the myoblasts were separated using 0.25% Trypsin and frozen in liquid nitrogen using freezing medium (30% FBS ,60% serum free media and 10% DMSO).

5 .Histological evaluation of scaffolds

Histological methods were used to evaluate the surface appearance of the extracellular matrix (ECM) components and basement membrane after decellularization. Decellularization was fully achieved, as evidenced by the elimination of cell nuclei and lipids, according to the results of the H&E staining. Moreover, the nuclei of the control group (intact tissue) are visible in the cell's corner thanks to the dark blue staining. General morphology was examined by staining scaffold histological slices and intact tissue with hematoxylin and eosin (H&E) and Masson trichrome staining. The process of cultured cells differentiating into muscle cells on scaffolds was also investigated, Suvarna et al(2012).

6 .Flow Cytometry Analysis

A linear amplifier was used to quantify CD90 for skeletal myoblasts utilizing flow cytometric analysis on a Mindray (BriCyte E6, China) flow cytometer. Both the forward and side scatter signals were recorded. Using a bandpass filter with excitation and emission wavelengths of 532 nm, logarithmically increased fluorescence signals from Phycoerythrin (PE) were found. The BriCyte E6 software was used to collect and evaluate the data; it can count the total number of cells in each sample without the need for additional beads, which lowers the BriCyte E6's operating costs, Nolan et al (2023).

Result

1- Scanning Electron Microscopic Analysis of the Scaffolds

The SEM micrographs revealed the presence of elastic fibers and collagen, two types of extracellular matrix, indicating that the scaffold's ultrastructure was retained and that all of its lipids had been removed (Fig.1 **A**). Moreover, the SEM micrograph of the unaltered tissue (control) demonstrated the high concentration of lipids in the tissue, which are visible as droplets in the SEM photos (Fig.1 **B**).

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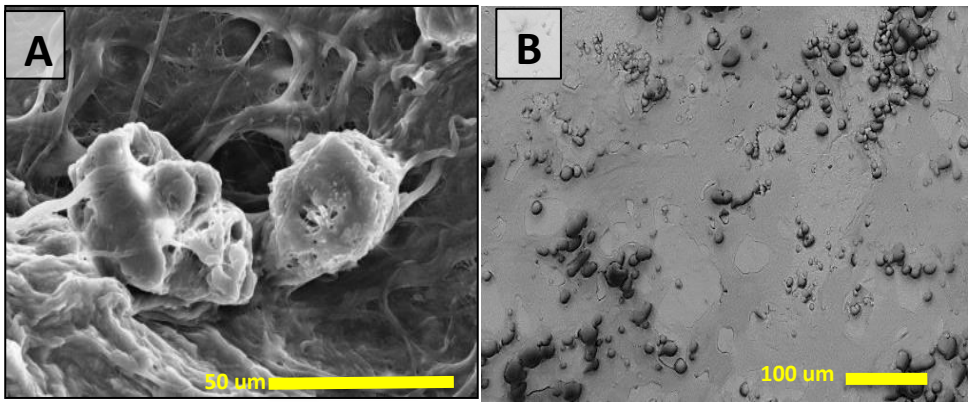

Figure 1. Ultra-structure micrographs (A. revealed the myoblasts cells, myotube and collagen fibers, B) showed after and before the lipid removed.

2- Mechanical tensile test

The greatest tensile stress (Stress) exerted by scaffolds was 1.78± 0.6 N, a substantial drop from 2.89± 0.4 N (p = 0.0003) for the control (intact tissue) (Fig. 2). At maximal stress, 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).

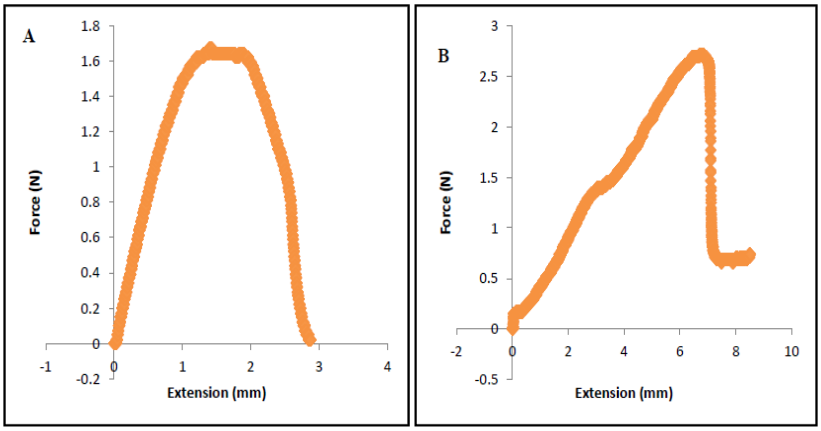

Figure. 2. Stress-strain curve of scaffold 1.78± 0.6 N (A) and control (Intact tissue) 2.89 ± 0.4 N (B).

3- Myoblast Isolation and Culture

As per the myoblast seeding, myoblasts were examined for their extended in vitro existence, proliferation, and fusion in the culture flask for 24, 72, and 120 hours prior to being seeded on matrix. As a result, a small group of myoblasts with a short spindle form were seen after 24 hours. At 72 hours, there were some long spindle-shaped myoblasts and a few multinuclear myotubes (Fig. 5); at 120 hours, there were still some long spindle-shaped myoblasts, but most of them were myotubes, as shown in Figures. 4A, B, C, D.

Myoblasts were cultivated in a culture after being planted on a scaffold. The seeded scaffolds were treated following the procedure outlined in above, after being cleaned with PBS on the first, second, and fifth day of culture. Specimens were then examined using a scanning electron microscope (LEO

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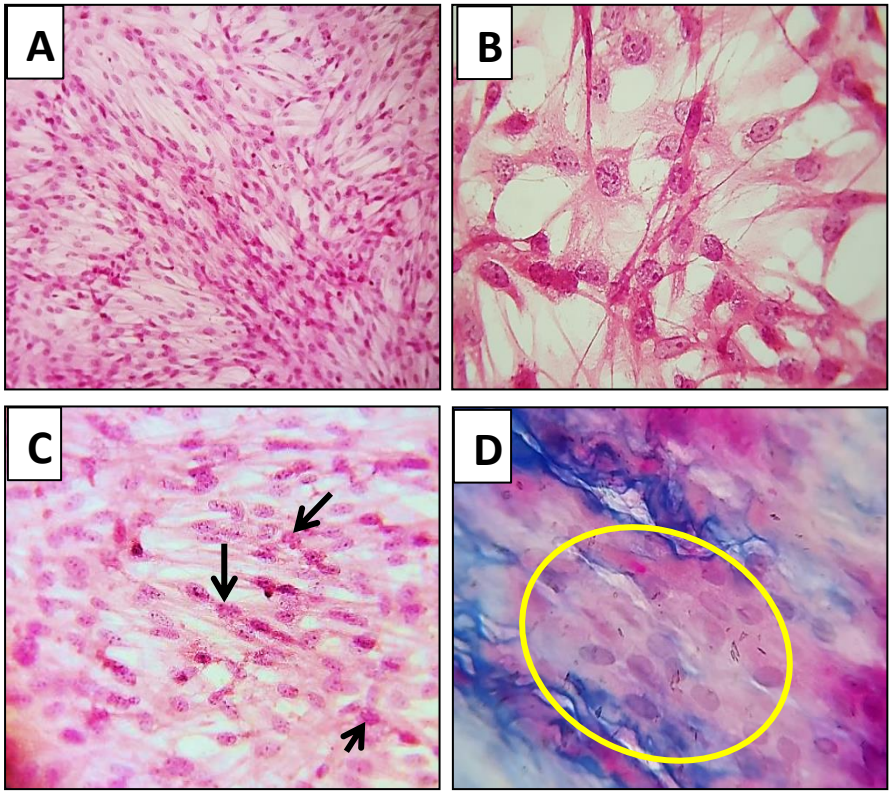
1430VP Germany High Voltage: 15 kV. Coating Metal: Au/Pd).



Photomicrography groups of myoblasts A) show with a short spindle form were seen after 24 hours, H & E stain 40x, B) at 72 hours, there were some long spindle-shaped myoblasts and a few multinuclear myotubes, H & E stain 400x, C) at 120 hours, there were still some long spindle-shaped myoblasts, but most of them were myotubes, H & E stain 100x, D) at 120 hours, there were obvious myotube (yellow circle) Masson Trichrome stain 400x.

4- Histological evaluation of scaffolds

Hematoxylin-eosin staining demonstrated that the seeded cells were adhered to the scaffolds and that they expanded and grew on their surface. Additionally, the scaffold allowed the cells to elongate and lengthen (Fig. 5 A, B, C, D)

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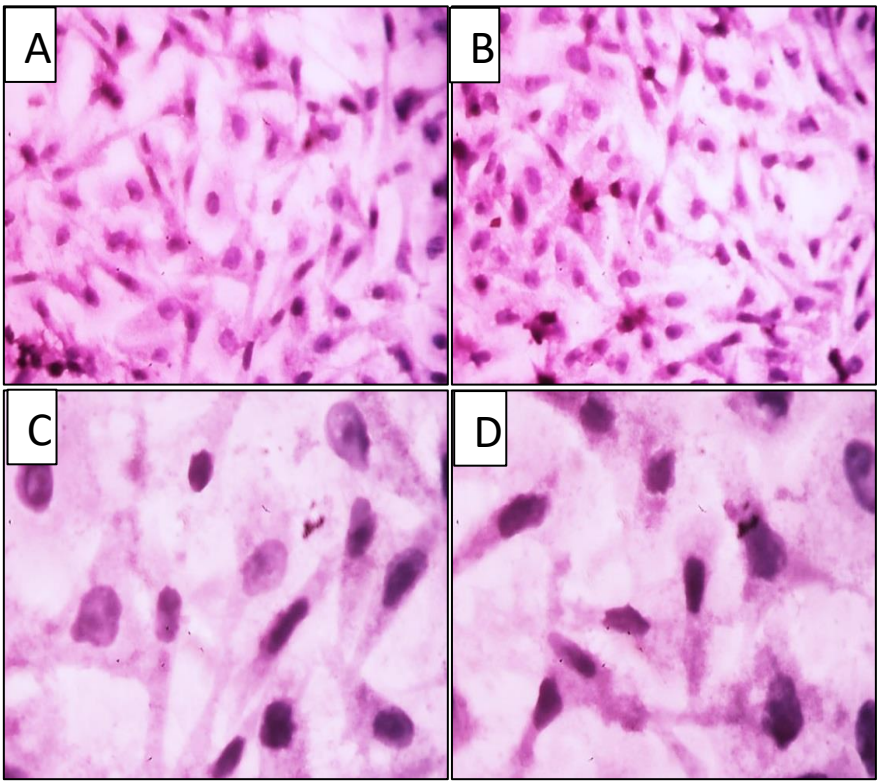

Figure 5, Stained with H&E. (A) and (B) at 400x, (C) and (D) at 1000x.

5- Flow Cytometry Analysis for Identification of Myoblast

To confirm the purity of the grown myoblasts, the product was analyzed using a Mindray flow cytometer (BriCyte E6, China). Approximately 95.33% of the cell clusters in CD90-positive samples automatically compensated, according to fluorescence analysis. (Figures. 6).

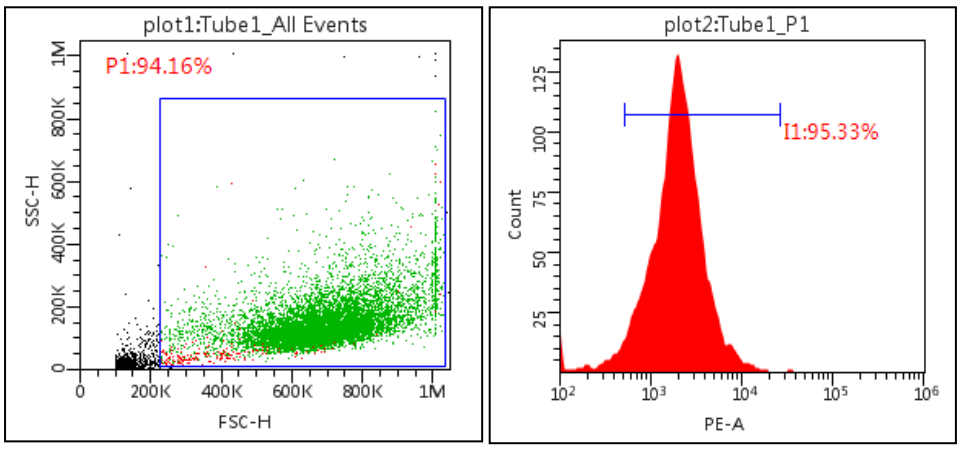

Figure 6: Flow cytometric histogram depicted Phycoerythrin (PE) conjugated positive myoblast

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(excited at 532-nm as a green) Note that the percentage of pure myoblast is 95.33% whereas non myoblast cells are 4.67% in flow cytometry analysis.

Discussion

A Light and Electron Microscopic Analysis of scaffold in addition to acting as a template for the three-dimensional structure of the growing tissue, biomaterials give the construct short-term mechanical stability Hutmacher (2001). Hence, choosing the best biomaterial scaffold for skeletal muscle tissue creation is essential in order to support myoblasts' proper differentiation and maturation into myotubes and myofibers. Many types of natural scaffolds have now been used to treat the muscle wall damage in experimental animal models. These include collagen Lai et al(2003), acellular dermal matrix Chung et al(2003), and swine small intestine submucosa badylak et al(2002). Detergent-enzymatically produced acellular matrices have the potential to serve as viable tissue replacements since they do not cause rejection reactions Roeder (1999) ,Parnigotto et al(2000), Parnigotto et al(2000) ,Sutherland (1996). In this work, a biological scaffold with mechanical and morphological characteristics similar to the original tissue was created using sheep omentum, These scaffolds appear to be entirely acellular, which might be the consequence of a little adjustment made to the preparation techniques utilized in Patrick et al(2023) earlier procedures combined with a low collagen density. However, scaffolds originating from sheep omentum had few cell remnants inside light collagen bundles; this may have been caused by the low density of the structural fiber components. Patrick et al(2023) have reported a different outcome. Nearly all cellular components were removed without ultrastructural signs of damage to fibrous components, according to light and electron microscopic investigations. Despite the fact that Huang et al (2004) study on the impact of an acellular biological tissue's degree of crosslinking on its pattern of tissue regeneration produced identical acellular results. However, they employed a unique method of cell extraction that was not utilized in this investigation. The serosal/inner surface of the omentum scaffold showed an uneven polygonal form structure with an irregular connective tissue appearance, as seen by an ultrastructural electron micrograph. According to Patrick et al(2000), there are several differences between the surface of freeze-dried omentum and fresh omentum, including the loss of the serosal layer, the exposure of underlying collagen bundles, the separation of individual collagen bundles, and the fibers, which have a well-defined, wavy appearance. The ability of satellite cells, or stem cells, to guide intrinsic cell programs linked to proliferation and differentiation is essential for skeletal muscle tissue creation. In recent years, the production of genetically modified myoblasts and muscle stem cells for transplantation has emerged as an intriguing and difficult therapy option for patients with muscle disorders Wu et al(2003), huard (2003),haider(2003). On the other hand, substantial muscle defects or sick areas may need to be repaired using big tissue grafts that have been heavily seeded with myoblasts. Consequently, the myoblasts were effectively separated and extracted from the primary cell culture that was produced by enzymatic digestion of thigh muscle fibers. Thus, by this effort, we were able to create cultures obtained from muscle satellite cells that included myoblasts and expressed the transcript factors required in the differentiation process of skeletal muscle cells , Chen and Goldhamer (2003). Furthermore, the fast rate of proliferation of cultured myoblasts means that it only takes three weeks to attain the ideal cell count for graft implantation. Thus, compared to earlier methods of isolating satellite cells Mazaro et al(2002), Van Wachem(1999), this myoblast isolation approach seems to provide better results; nevertheless, data published by , Conconi et al (2005) seem to support this theory.

SEM electron micrographs of the in vitro results showed that the sheep omentum scaffold could support myoblast differentiation and growth. Within 24 hours, a small number of myoblasts started to cover the whole surface of the scaffold and merged into a myotube. The myoblast continues to fuse and generate a series of evenly arranged myotubes between the third and fifth days after seeding, but by the fifth day, densely There were also detected acked myotubes with morphology resembling myofibers. These findings were almost in agreement with those published by yan(2006), whose investigation concerned tissue engineering of skeletal muscle utilizing aligned collagen gel covered tissue flask. Van Wachem(1999), have reported a similar outcome using autologous myoblast-seeded homologous muscle acellular matrix as a tissue-engineering technique for abdominal wall defect healing. Flow

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cytometric analysis was used to determine the percentages of myoblasts, and this analysis indicates that over 95.33% of the skeletal myoblast cells that were separated had developed into myogenic cells, according to Lai et al(2003) over 80% of skeletal muscle cells that were isolated had a myogenic character. This technique was important in proving the high purity of the myoblast cells using the CD90 marker Alfonso (2011).

Conclusion

We conclude from the results obtained that the manufactured scaffold was appropriately biocompatible with the cells, and the cell extraction process also had a good yield in terms of the number of cells obtained. It was also shown that the transplanted cells grew well on the scaffold, which indicates the positive effect of the scaffold on the cells.

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