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Soft tissue regeneration in animal models using grafts from adipose mesenchymal stem cells and peripheral blood fibrin gel

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Abstract. – OBJECTIVE: Our study aimed to evaluate the effect of soft tissue regeneration in nude mice using grafts made from the combination of adipocytes from fat tissue mesenchymal stem cells and fibrin gel from peripheral blood.

MATERIALS AND METHODS: Mesenchymal stem cells were isolated from adipose tissue and identified according to ISCT criteria. The scaffold used was fibrin obtained from peripheral blood. The grafts in this study were generated by transferring mesenchymal stem cells onto a fibrin scaffold. Two types of grafts, the research sample (fibrin scaffold containing adipocytes differentiated from mesenchymal stem cells) and the control sample (fibrin scaffold only), were grafted under the dorsal skin of the same mouse. After each research period, samples were collected and evaluated by histological methods to observe the existence and growth of cells inside the grafts.

RESULTS: The results showed that the study group's graft integrated better within the tissue when compared with the control group. In addition, the grafts in the study group showed the presence of cells with characteristic morphology of adipocytes one week after transplantation. In contrast, control samples showed dimorphous shapes and features mainly composed of non-homogenous fragments.

CONCLUSIONS: These initial conclusions might be considered a first step in generating safe bio-compatible engineered grafts specifically usable in post-traumatic tissue regeneration procedures.

Key Words:
Adipose-derived mesenchymal stem cell, Fibrin scaffold, Soft tissue regeneration.

Introduction

Autologous fat grafting is the gold standard for repairing and regenerating damaged soft tissue. Despite the great achievements there is still the need to adopt better strategies to obtain higher-quality graft materials for these urges. Adipose-like bioengineering grafts are a pressing concern worldwide, and new results[1] have been showing enough potential to replace pure

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autologous adipose tissue. The current study proposed the creation of a fibrin tissue substrate as a scaffold to locate stem cells for a ready-to-go bio-compatible adipose scaffold to use in clinical reconstructive procedures[2-8]. Our team has recently been designing new 3D biomaterial by combining human autologous adipose-derived mesenchymal stem cells (AT-MSCs) inserted onto a fibrin-gel scaffold obtained from human autologous peripheral blood. Although many studies[9-15] have described the unique features of AT-MSCs in regenerative medicine, the interest in fibrin gel and its use in clinical procedures, though it dates back to the late 19th century, still remains to be fully disclosed. This study highlighted fibrin's unique features as a highly polyhedric biomaterial capable of containing different types of cells and stem cells to regenerate damaged soft tissues[16-24]. The study involved the use of nude mice which received subcutaneous implantation of graft composed of AT-MSCs and fibrin gel substrate, and the fibrin gel only both positioned on either side of the back. The follow-up time to pair samples on mice for two weeks, then the grafts on research mice will be collected at 2 study time points. Twelve nude mice were used in the study and were divided into two groups, each group consisted of 6 mice. Mice were evaluated for the first time during the first week after the transplantation and divided in two experimental groups, the graft group (total of 3 mice, graft with stem cells) and the control group (total of 3 mice, graft without stem cells). In the second week, mice were evaluated on two experimental groups, the graft group (total of 3 mice, graft with stem cells) and the control group (total of 3 mice, graft without stem cells). This study revealed that AT-MSCs and fibrin grafts showed quite unique biological and physical properties that mimic the native extracellular niche (ECM niche), crucial for stem cell adhesion, growth, proliferation, and differentiation along particular lineages. This study also focused on the current and future applications of AT-MSCs and fibrin gel grafts, which may represent a new perspective for further potential explorations in tissue engineering for clinical applications.

Materials and Methods

Materials

Fat tissue was obtained from the fat tissue of healthy donors through liposuction in the operating room and after they gave consent allowing their use for research purposes. They have been used to isolate and cultivate mesenchymal stem cells. Plasma was collected from the Department of Hematology, following the hospital's guidelines and regulations. The plasma was then used to collect fibrin to generate the scaffolds to home adipose tissue mesenchymal stem cells[24-26]. The mice used in the experiment are nude, male, about 6-8 weeks old, and weighing about 20-35 grams.

Methods

Our team used experimental and descriptive research methods to conduct this study. The total number of evaluated nude mice was twelve (following the regulations of the Vietnamese Animal Regulatory Act); the mice were divided into two experimental groups. Group 1 included mice transplanted with engineered adipose tissue grafts (research samples) containing differentiated adipocytes from AT-MSCs. Group 2 included mice grafted with fibrin scaffold samples (control samples). In each experimental group, mice have evaluated at two time points 1 week (3 mice total) and two weeks (3 mice total). Each mouse received grafts on both the right and left sides of the back (Fibrin scaffold), respectively. The total number of repetitions was three times for each session. At each point in the research process, mice of the different groups were sacrificed to collect grafts and fibrin samples, and the specimens were collected to evaluate by H&E stain. The research contents carry out according to the following steps: (i) isolation, identification, and differentiation of AT-MSCs from the adipose tissue; (ii) construction of the fibrin scaffolds; (iii) transfer of AT-MSCs onto fibrin scaffolds and differentiation to adipocyte[3,11,26]; (iv) the grafts were collected and inserted under the skin of the nude mice back at day seven. At the study time points, grafts were collected and histologically analyzed. The grafts' cellular morphology and structure were carefully observed under an optical microscope to assess the structural integrity of the graft and the presence of adipocytes. Finally, all data were analyzed using appropriate methods for each study section.

Isolation, Culture, and Identification of Mesenchymal Stem Cells from Human Adipose Tissue

Fat tissue (about 20.0-30.0 ml) was transferred into sterilized Conical Centrifuge Tubes (Falcon-

™, 50 mL, Thermo Fisher Scientific, Waltham, MA, USA). Then, in each tube, these tubes added a mixture of Dispase-Collagenase enzymes, with a volume ratio of 1.0 (fat sample): 2.0 (enzymes), and incubated at 37°C for 90 min. After incubation, these tubes were centrifuged to collect the cell fraction deposited at the bottom of these tubes. Then, this cell fraction was added to a cell culture medium (StemPro™ MSC SFM, Gibco™, Grand Island, NJ, USA) for cell suspension, and continues to take cells were filtered through a Cell Strainer (Falcon™, 70 µM, Thermo Fisher Scientific, Waltham, MA, USA) to collect the mesenchymal stem cells. An erythrocyte lysis solution was used to remove red blood cells and then centrifuged to collect cell residues. Trypan blue solution was used to count the number of cells in the Neubauer counting chamber. Cells were immerged into the StemPro™ medium supplemented with Penicillin-Streptomycin (10,000 U/mL) (Gibco™, Grand Island, NJ, USA) in a T-25 cm^2 flask (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C and incubated at 5% CO_2. Stem cells were cultured until the second generation and identified as mesenchymal stem cells according to ISCT criteria[7].

Preparation of Fibrin Scaffold

We generated the fibrin scaffold by combining fibrinogen and thrombin from healthy consenting donors obtained from 5.0 ml of human peripheral blood. This blood was divided into two equal parts, 2.5 ml each tube; the first tube for collected fibrinogen, and the part residue for received thrombin. The fibrin scaffold was created by mixing fibrinogen and thrombin in an equal volume ratio of 1.0:1.0 (v/v).

Procedure to Obtain Fibrinogen

The blood was centrifuged at 3,000 rpm for 5.0 minutes to collect the plasma fraction[6,10]. Approximately 10.0 ml of plasma was collected and centrifuged at 3,000 rpm for 5.0 minutes. This plasma was then sterilized as a scaffold for cell culture by filtration through Minisart® Syringe Filters (0.20 µm, Sartorius, Goettingen, Germany). This plasma was incubated in a refrigerator at 4.0°C for 1 hour and then transferred to another fridge at -20°C overnight.

Procedure to Obtain Thrombin

The frozen plasma thawed at 4°C. Then, this plasma was supplemented with a sterilized PBS solution composed of a ratio of 1.0 ml plasma: 9.0 ml PBS solution (v/v). Acetic acid (1%) was added to adjust the pH to 5.3[16]. This solution was left at room temperature for 30 min to give a white precipitate and centrifuged at 3,000 rpm for 5 min to collect this sediment at the bottom of the tube. Then, PBS solution (2.0 ml) was added to make a suspension solution. The Na_2CO_3 solution (0.1 M) was used to adjust the pH to 7.0. The tube was placed in a thermostatic bath at 37°C for 15 minutes and added $CaCl_2$ solution (0.01 M) was to form a white gel. This white gel was then removed, and the remaining liquid was in the tube as a thrombin solution. Thrombin was stored at –20°C and used within three months.

The Generation of Bio-Scaffold by Combining AT-MSCs Adipocytes and Fibrin Gel

The AT-MSCs were harvested at the second subculture and inserted into the fibrin gel. Approximately 10^5 cells were mixed with 1.0 ml of fibrinogen solution and 1.0 ml of thrombin solution to create grafts. Then, these grafts were cultured in the StemPro™ medium. After one day of culture, a new medium was replaced with StemPro™ Adipogenesis Differentiation Kit (Gibco™, Grand Island, NJ, USA) to induce AT-MSCs differentiation into adipocytes. After seven days, these grafts were ready for transplantation into mice.

Evaluation of the Survival and Development of Grafts in a Nude Mice Model

All graft and control samples were surgically collected. Specimens were carefully observed and evaluated for clinical signs. The pieces were then subjected to histology staining procedure (H&E staining) to assess the structural integrity of the tissue and the presence of adipocytes in the graft and control samples (Figure 1).

Statistical Analysis

Data expressed as mean ± standard error of the mean (SE). Statistical analysis was performed using the *t*-test to compare the data pairs in the results of the two groups if they are typically distributed, p-value < 0.05, indicating that the results are statistically significant. All experiments were performed in triple procedure.

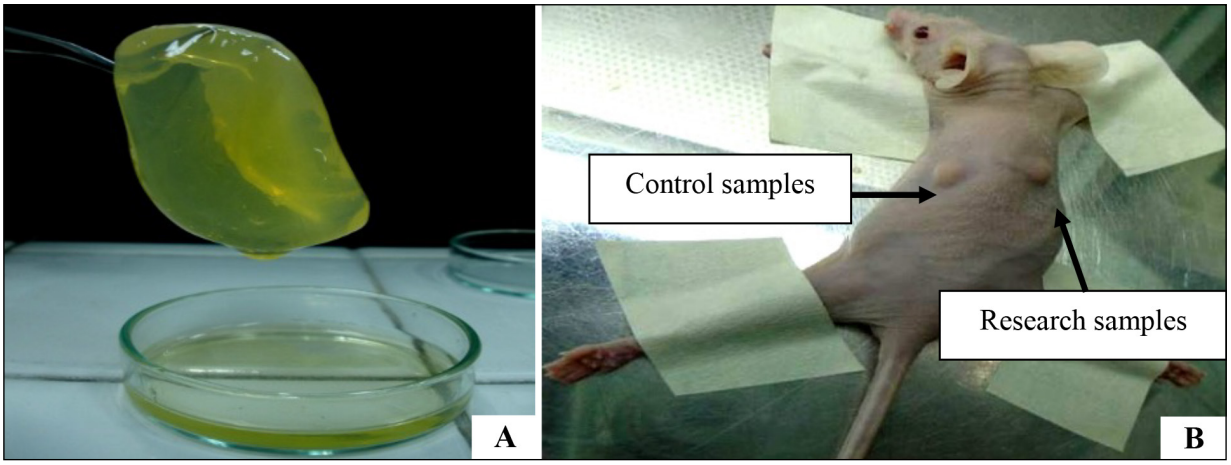

Figure 1. Evaluation of soft tissue grafts. **A,** Soft tissue graft. **B,** Each model received two grafts control and research samples. We inserted the research samples to the right-back side of the mouse. And the control samples were grafted to the left-back side of the mouse.

Results

AT-MSCs from Human Adipose Tissue

The results showed that AT-MSCs were phenotypically negative for some markers, such as CD14, CD45, and HLA-DR. These cells have phenotypically positive markers, such as CD13, CD44, CD73, CD90, and CD105. We obtained stem cells from donor adipose tissues using appropriate culture procedures, which responded to the International Society for Cellular Therapy (ISCT) criteria for identification as adipose-derived mesenchymal stem cells. (i) AT-MSCs must adhere to the bottom of cell culture flasks; (ii) AT-MSCs must retain the multipotency trait; (iii) AT-MSCs must express a few specific CD markers at least CD73, CD90, and CD105. The results confirmed the plasticity features of MSCs that differentiate into adipocytes, osteoblasts, and chondroblasts in vitro under appropriate culture and kit conditions.

The AT-MSCs growth in the second subculture responded to the criteria of the ISCT. The cells showed an elongated shape, resembling fibroblast-like cells. These cells were adhesive to the bottom of the culture flasks. These cells were able to differentiate into osteoblasts, chondrocytes, and adipocytes (Figure 2) and display important mesenchymal stem cell markers such as CD73, CD90, and CD105 when assessed by Flow cytometry (Figure 3). These cells were then homed into the fibrin scaffold and induced to differentiate into the adipose-tissue scaffold, eventually grafted in nude mice.

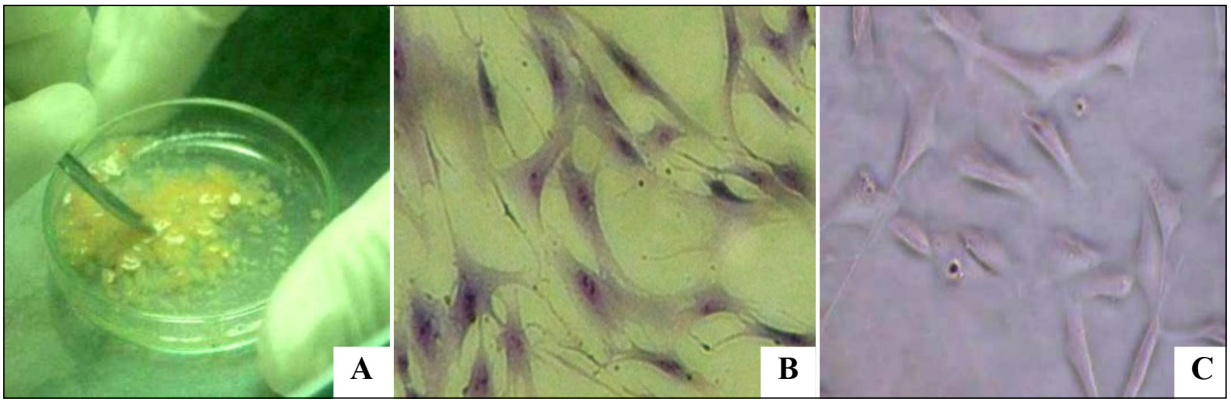

Figure 2. Isolation and culture of stem cells from adipose tissue. **A,** Fat tissue process. **B,** Cells after seven days of culture, shaped like fibroblasts (Giemsa staining), 20×. **C,** Cells observe by a phase-contrast microscope. They have an elongated shape that adheres to the bottom of the flask, 20×.

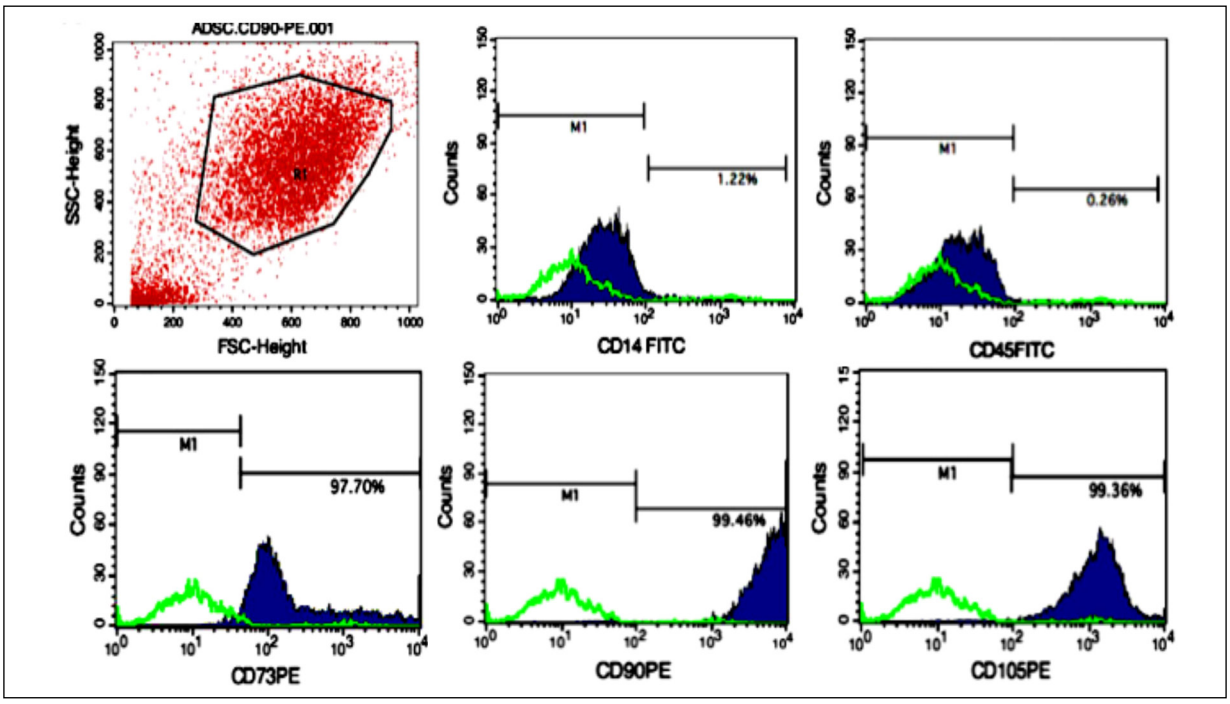

Figure 3. Results of evaluation by flow cytometry to identify mesenchymal stem cells. AT-MSCs were phenotypically negative for some markers like CD14, CD45, and HLA-DR and positive for some feature markers, such as CD13, CD44, CD73, CD90, and CD105.

Result of Obtaining Fibrin Used as Scaffolds

The fibrin gel obtained from the combination of fibrinogen and thrombin showed the traits of a well-structured bio-scaffold composed of a porous internal architecture with interconnecting micro tunnels, an ideal home to accommodate stem cells (Figure 4). The cells were effectively adhered and developed within the scaffold, capable of changing the internal morphology, as shown in Figure 5. The appearance of fat droplets within the cells confirmed the presence of adipocytes. These fat droplets tended to become more numerous as the culture time increased to form consistent clumps.

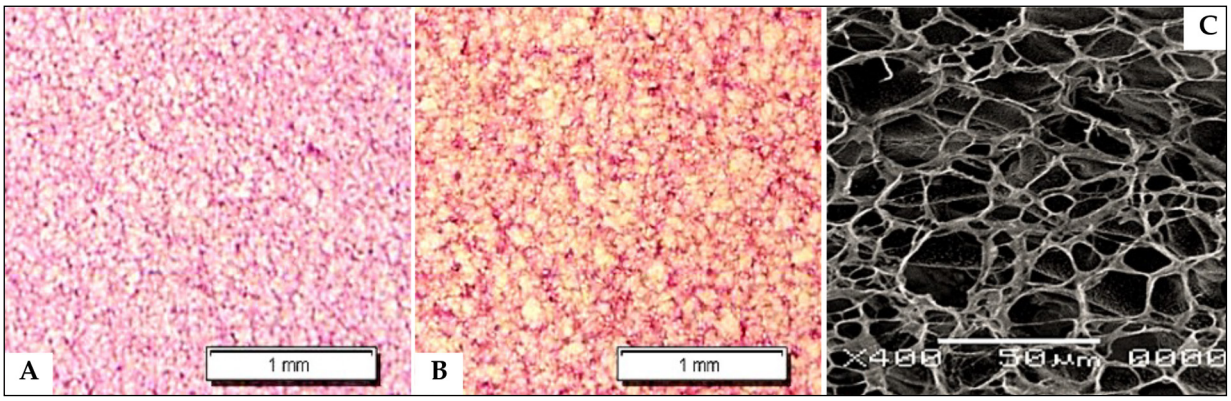

Figure 4. Research results to create fibrin scaffold used for cell culture. **A,** Observe the fibrin scaffold under an optical microscope after being stained with H&E (10×). **B,** Histological results show that the fibrin scaffold has a tubular structure connected (20×). **C,** Fibrin fibers have a branching structure, and these fibers are connected, which helps the cell adhere and grow on the fibrin scaffold (SEM, ×400, 50 µm).

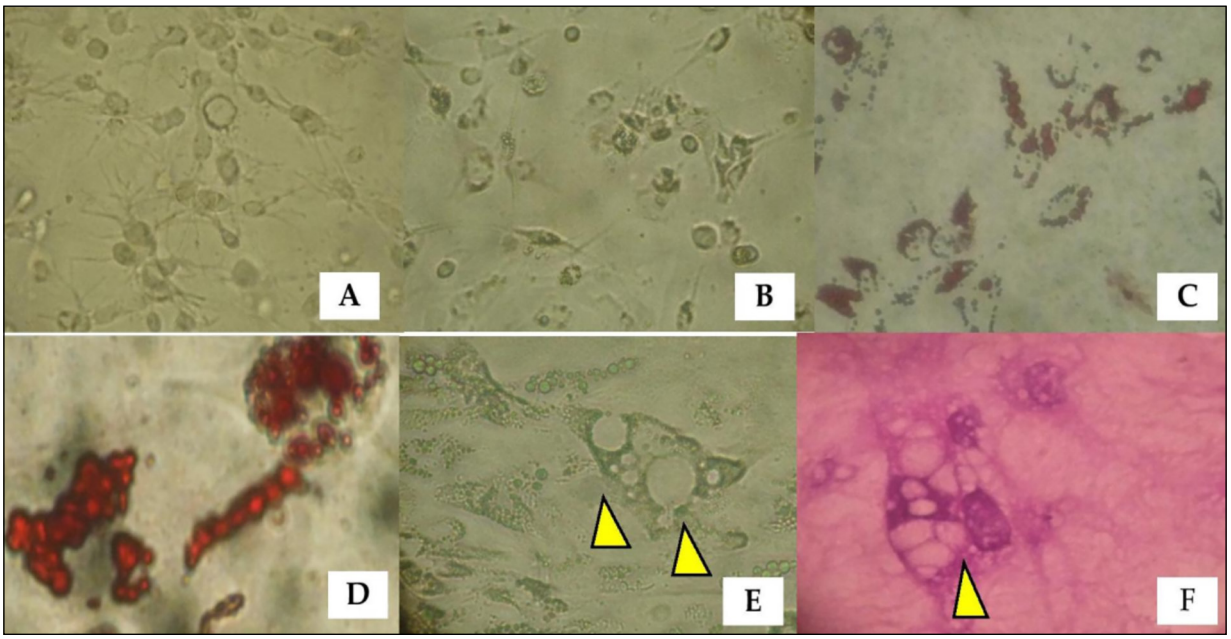

Figure 5. Results of evaluation of adipose tissue engineering grafts. **A,** and **B,** The MSCs differentiated into adipocytes on the surface and inner fibrin scaffolds (10×). **C,** and **D,** Oil Red O staining results for adipocytes have differentiated from MSCs on the scaffold fibrin (10×, 20×). **E,** and **F,** Histological staining H&E, showing cells growing inside scaffold fibrin. Evaluation of histological staining showed that the cells differentiated into adipocytes. Cells that have demonstrated the properties of fat cells are lipid storage (yellow arrowheads) (10×, 40×).

Fat droplets are considered an essential criterion to assess the presence of adipocyte phenotypes, as stated by the ISCT criteria for identification as adipose-derived mesenchymal stem cells. These newly generated adipocytes were then homed into the fibrin scaffold to create fatty tissue to be grafted under the skin of nude mice. The team evaluated the graft caused by the introduction of AT-MSCs into the fibrin scaffold (Figure 5).

Direct observation under the inverted microscope showed that cells could firmly home within grafts (Figure 5A). The cells were rhombus-shaped with dendrites. The AT-MSCs developed and grew within the fibrin gel showing a good state of differentiation to adipocytes due to using a suitable cell differentiation medium, confirmed by fat-droplet formation and accumulation (Figure 5B). The adipocyte favored the staining procedure with Oil Red O (Figure 5C-5D). The AT-MSCs cultured and differentiated adipocytes on the fibrin scaffold were directly observed under the phase inversion microscope (Figure 5E) and on the histological slides (Figure 5F). The observational results showed that the cells had fat droplets around the cell nucleus (Figure 5F), demonstrating that AT-MSCs successfully differentiated into adipocytes *in vitro*.

Results of Evaluating the Survival and Growth of Cells Inside the Grafts in Nude Mice

Research samples (grafts containing differentiated adipocytes from MCSs)

The study samples were surgically removed and assessed. Observation at the surgical site showed no signs of inflammation or abnormalities. The grafts were smaller in size, suggestive of well absorption process within the host (Figure 6A).

The histology results confirmed that the structure of the study group grafts remained integral and homogeneous. The observation revealed the presence of a few inflammatory cells and a high presence of adipocytes, which were the cells we expected to appear. The result proved the excellent state of cells one week after transplantation showing that the graft integrated well into the surrounding host tissue. The grafts were almost absorbed within the host tissue two weeks after transplantation.

The Control Samples (Fibrin-Gel Scaffolds Without Cells)

Control samples were surgically re-acquired and histologically evaluated. After one week

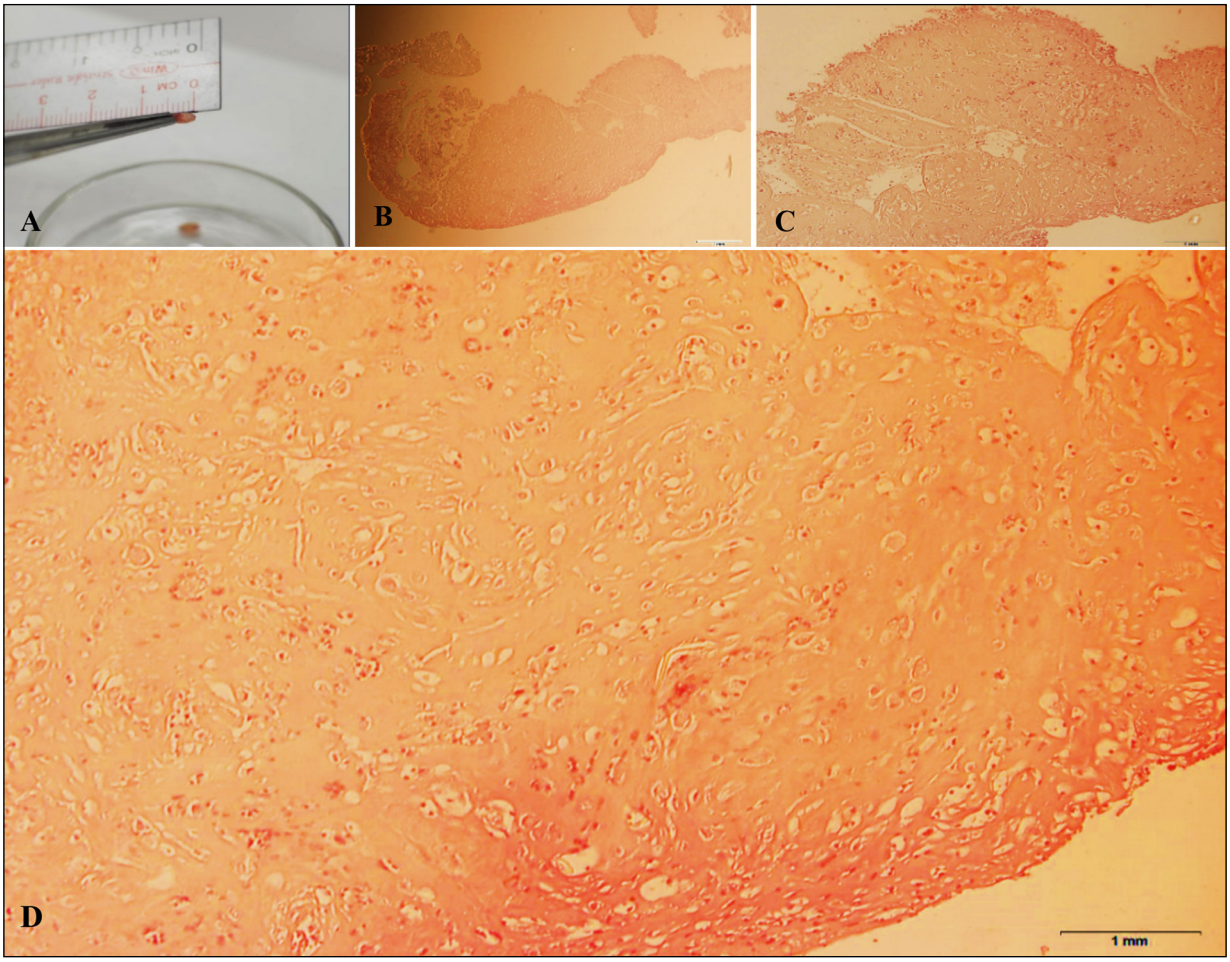

Figure 6. Evaluation of histological results on grafts (the research samples). **A,** Surgery to collect grafts back. **B,** The graft was processed into a slide and stained with H&E dye (4×). **C,** Observe the specimen at the 10× objective (10×). **D,** The graft contains a lot of fat cells (*black arrow*) and blood vessels (blue triangle) (40×). Two weeks after grafting, we surgically collect all remaining. The absence of inflammation processes and abnormal signs were confirmed at the surgical site. At this stage, the graft was fully integrated within the host tissue.

of implantation, the models showed no signs of inflammation or other abnormalities at the surgical site. However, the evaluation results show that the fibrin gel has a change in size; namely, the size of fibrin is smaller than the original size before grafting under the dorsal skin of mice. Histological results showed that the graft structure degenerated into smaller, amorphous parts with many blood vessels infiltrating and the appearance of inflammatory cells; no fat cells were present in the observed tissue structure. The fibrin gel was totally absorbed into the host tissue two weeks after implantation. The results after two weeks of transplantation were similar to those of the study sample, and we do not have histological data at this point (Figure 7).

Discussion

The multipotent AT-MSCs are involved in the regenerative pathways participating in different levels, directly generating new adult clones, same as those of the damaged tissues and allowing the derivation of other functional cells and growth factors for regenerative purposes. Stemness of the AT-MSCs obtained in culture regarding proliferation, and multiple lineage phenotype is essential for inducing these stem cells to specific lineage differentiation. In this study, primary characterization using surface markers and lineage phenotype confirmed the suitable quality of AT-MSCs before attempting to assess their reparative capacities. Adipocytes derived from mesenchymal stem cells using exogenous differentiated induc-

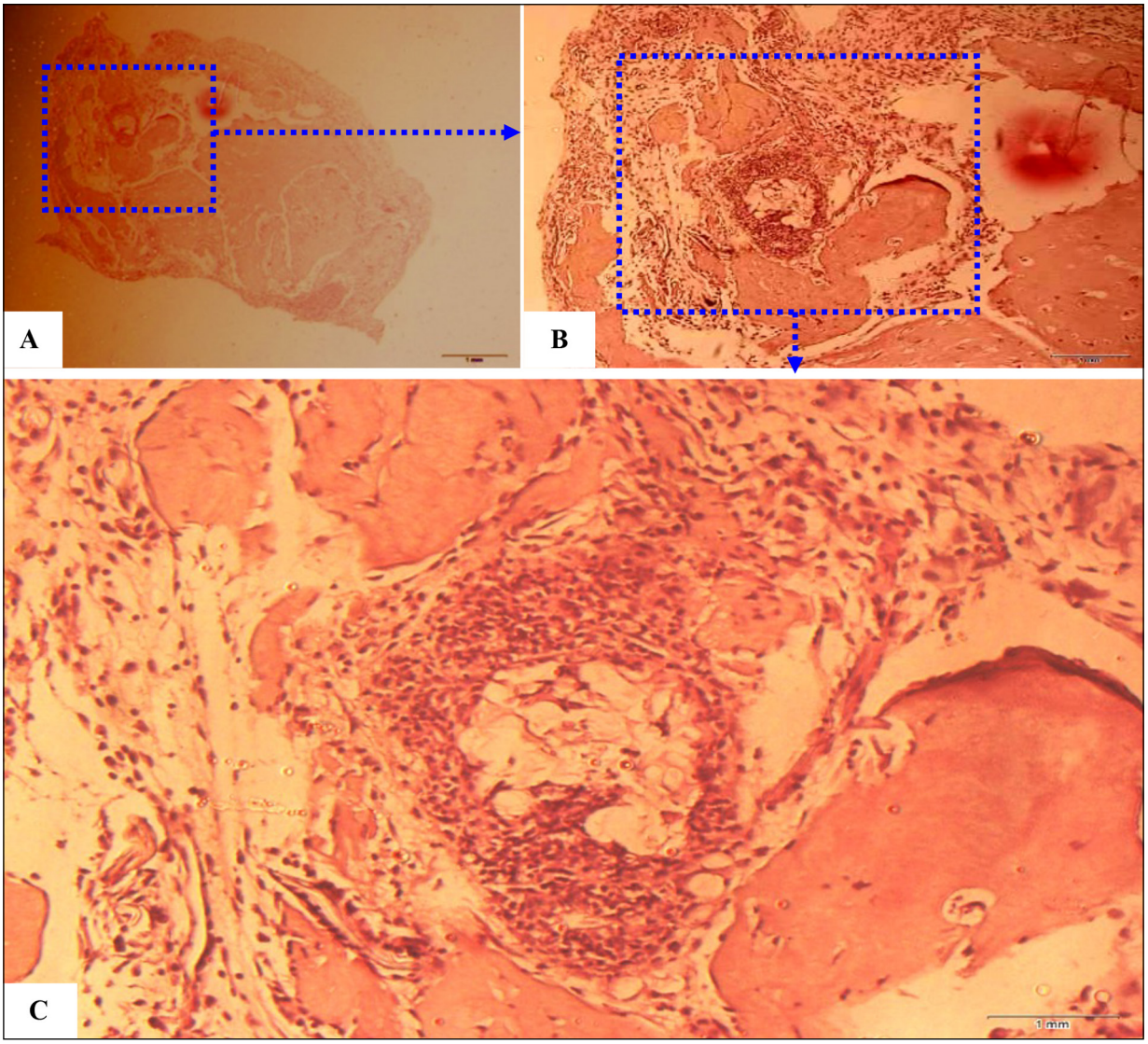

Figure 7. Evaluation of histological results on fibrin scaffold (the control samples). **A,** The fibrin scaffold sample was collected and histologically performed (H&E staining) (4×). **B-C,** Observe the results at the objective (10×) and (40×), respectively. The fibrin samples are not intact (blue squares), they divide into many lobes, and many blood vessels are present. No adipocytes existed, but many inflammatory cells were inside the fibrin matrix.

ers often in transient changes with the upregulation of a few adipose markers. Exploiting the potential of growth factors naturally included in the fibrin scaffold as natural inducers for stable and diverse differentiation of AT-MSC soft tissue cells opens several opportunities. In soft tissue regeneration, converting mesodermal origin AT-MSCs into regular, highly differentiated lineage tissue (muscles, tendons, vessels, heart, etc.) is a significant challenge[27-33].

The knowledge that the fibrin matrix supports the differentiation and proliferation of different phenotypes within the separate compartments of the soft tissues prompted the design of a cell-specific fibrin niche for the differentiation of AT-MSCs to these lineage cells[31-33]. The study highlights that the AT-MSCs could be differentiated into different proliferating progenitor stages and then into adipocytes. The behavior and characteristics of each cell type suggest the presence of a biomimetic process that makes these steady and stable changes in well-organized and bio-compatible soft tissue.

Fibrin has been studied[6,10] and used in clinical procedures as a coagulation agent and bio-glue for decades. Until now, fibrin has been discovered

to have a new potential application in tissue engineering as a scaffold for culturing many types of cells to create many different types of tissue engineering grafts[10,24-33]. Therefore, we designed this study to develop soft tissue grafts based on the patient's peripheral blood and adipose tissue to have grafts with structures similar to autologous adipose tissue. These grafts open therapeutic prospects for traumatic and postoperative soft tissue lesions (cancer). This research helps to develop new technical grafts that can use safely and effectively.

The fibrin matrix also supports the differentiation of MSCs into adipocytes in adipocyte differentiating medium. The outcomes showed that the final product is highly similar to human adipose tissue, with adipocytes growing within the fibrin matrix[9,14,23,24,30-32]. The experiments using exogenous differentiation inducers inhibitors have proven that specific signaling promotes AT-MSCs proliferation, developing into different cell phenotypes comprising adipocytes, mimicking the body's pathway. Thus, the coordinated action of AT-MSCs and fibrin gel signaling within the host's tissue microenvironment could be the basis for soft-tissue repairing and new cell formation[31-35].

Adipocytes were identified and morphologically assessed by observation under an inverted microscope. In addition, these cells were evaluated histologically, and fat droplets were found within the cytoplasm of the cells. We evaluated graft survival and growth in nude mice during the first week after transplantation, and the test confirmed that these cells retained the characteristics of adipocytes (Figure 6-7). The grafts did not show any unwanted reaction and were integrated correctly and absorbed within the host tissue.

Biopsies performed on either graft samples "Research or Control" showed safety and excellent integration within the host tissues; however, the research samples showed longer and better stability within the host tissues. Histological results of the graft showed the presence of fat cells in the tissue structure, and the cells increased and matured at the time of transplantation. This evaluation result is consistent with the use of Oil Red O dye to identify adipocytes because following the differentiation process of AT-MSCs into adipocytes will gradually accumulate lipid droplets inside the cells and more and more with time the induction of differentiation. We assume that the constitutive components of fibrin gel and AT-MSC signaling are elicited and influence differentiation; however, graft proliferation and reabsorption have yet to be carefully observed and evaluated because the study time is not well-controlled. Conversely, control samples generated empty disorganized structures that progressively deteriorated faster. This study may contribute to a "ready-to-go" proposal in a critical scenario of post-traumatic and post-surgical events, explicitly in breast tissue reconstruction and complex plastic surgery resections.

Conclusions

We know this study still has to solve some substantial limitations linked to the low number of cases and data with more convincing results. We are also conscious of the need for deeper investigative analysis to reach more reliable and solid outcomes. These observations include the essential prerequisite for verifying the optimal time for graft harvesting and better understanding cells' performance and their regenerative potential within the grafts. The density of cells and the number of fat cells within the graft are also crucial to consider. In this study, we found that some limitations need to improve in future studies: (1) degradation and absorption quickly when using fibrin as a cell scaffold; (2) evaluate the potential for heterotopic ossification that may induce by ectopic lipodystrophy adipocytes and the degree of toxicity; (3), difficulty in quantification and identification of adipocytes within the fibrin structure *in vitro* as well as in transplantation in mouse models. Nonetheless, these results are a promising research direction in adipose tissue engineering, especially considering their potential in soft tissue regeneration and plastic surgery[29,30].

Conflict of Interest

The Authors declare that they have no conflict of interests.

Authors' Contribution

Conceptualization, T.D.H., H.K.N., B.T.N., T.C.T., C.I.G., A.M.I., M.G.B., V.H.P. and F.I.; methodology, H.L.B.T., E.M.S., T.A.N.H., A.M.I., F.I., G.R., C.I.G., and G.D.; software, R.L., P.D., M.G.B. and F.I.; validation, F.I., C.I.G., V.H.P., M.G.B. and T.C.T.; formal analysis, F.I., C.I.G., K.C.D.N., A.M., V.H.P. and T.C.T.; investigation, T.D.H., H.K.N., B.T.N., M.G.B., T.C.T., and F.I.; resources, T.C.T., G.D., K.C.D.N., V.H.P. and F.I.; data curation, F.I. and C.G.I.; writing original draft preparation, F.I., A.M.I.,

C.I.G., V.H.P., G.R. and T.C.T.; writing review and editing, F.I., A.M.I., C.I.G., M.G.B., V.H.P. and T.C.T.; visualization, F.I., A.M., G.D., C.I.G., V.H.P. and T.D.H.; supervision, F.I., C.I.G. and T.C.T.; project administration, F.I., B.R., A.M.I., C.I.G. and K.C.D.N. All authors have read and agreed to the published version of the manuscript.

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Ethics Approval

The entire study has conducted according to Vietnamese Ministry of Health guidelines. All procedures have been followed the declaration of Helsinki for the reuse of human biospecimens in scientific research and the human study protocols approved by the Research Ethics Committee in Biomedical Research of Pham Ngoc Thach Medical University with number: n496IRB-VN01013.

Informed Consent

Informed consent was obtained from all subjects involved in the study.

Availability of Data and Materials

All experimental data to support the findings of this study are available contacting the corresponding author upon request.

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