Journal of Dermatological Treatment

ISSN: 0954-6634 (Print) 1471-1753 (Online) Journal homepage: www.tandfonline.com/journals/ijdt20

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To cite this article: Gamze Tumentemur, Elif Ganime Aygun, Bulut Yurtsever, Didem Cakirsoy & Ercument Ovali (2025) Effect of amniotic fluid on hair follicle growth, Journal of Dermatological Treatment, 36:1, 2451389, DOI: 10.1080/09546634.2025.2451389

To link to this article: https://doi.org/10.1080/09546634.2025.2451389

Published online: 19 Jan 2025.

JOURNAL OF DERMATOLOGICAL TREATMENT
2025, VOL. 36, NO. 1, 2451389
https://doi.org/10.1080/09546634.2025.2451389

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Effect of amniotic fluid on hair follicle growth

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ABSTRACT

Purpose: Human amniotic fluid stem cells (hAFSCs) have shown significant regenerative potential in treating hair loss, wound healing, and tissue repair. This study aims to evaluate the effects of human amniotic fluid (hAF) on hair follicle (HF) regeneration and immune system modulation.

Materials and Methods: The hAF used was pooled, acellular, and gamma-irradiated to standardize its contents and enhance its stability. Both irradiated (FAFI) and non-irradiated (FAF) hAF were assessed for their efficacy and safety in promoting hair growth and modulating immune responses in a rat model of hair loss. The study examined HF regeneration, transition to the anagen phase, and macrophage polarization from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype.

Results: Both FAF and FAFI treatments significantly increased HF density, with FAFI exhibiting enhanced effects. Histological analysis demonstrated improved HF regeneration, increased M2 macrophages, and reduced collagen fiber deposition in treated areas. Gamma irradiation likely improved the efficacy of FAFI by stabilizing active components and inhibiting protease activity.

Conclusions: Irradiated hAF is a safe and effective therapeutic candidate for alopecia and HF growth disorders. These findings support further evaluation of hAF in clinical trials to validate its potential for hair regeneration therapies.

ARTICLE HISTORY
Received 17 October 2024
Accepted 2 January 2025

KEYWORDS
Amniotic fluid; freezing; pooling; gamma irradiation; hair follicle; macrophage

Introduction

Hair loss (alopecia) is a prevalent condition characterized by the partial or complete absence of hair, which can affect various parts of the body. Alopecia can be broadly classified into two main categories: scarring and non-scarring. Scarring alopecia involves irreversible destruction of hair follicles, resulting in permanent hair loss. In contrast, non-scarring alopecia alters the capillary cycle, but the hair follicles remain intact, allowing the possibility of hair regrowth (1). Non-scarring forms of hair loss include conditions such as alopecia areata (AA), trichotillomania (TT), tinea capitis (TC), androgenetic alopecia (AGA), telogen effluvium, and anagen effluvium. On the other hand, scarring forms of hair loss include lymphocytic scarring alopecias and neutrophilic scarring alopecias (1,2). Although the exact causes of alopecia are still unclear, numerous studies indicate that environmental, immunological, and genetic factors may influence its development and progression (3).

Alopecia areata (AA) is an autoimmune hair disorder (4), whereas androgenic alopecia (AGA) occurs with the effect of androgens and can be seen progressing in males. AA may be treated with oral or topical drugs, or surgical options. Topical drug treatment includes corticosteroids, minoxidil, and immunotherapy (5). Contact immunotherapy with diphenylcyclopropenone is mainly used for alopecia (6). However, these drugs have side effects and limited therapeutic uses (7). Recently, many cytokine-targeted drugs have been reported to be effective for AA (8). A hair follicle (HF) is a mini-organ containing epidermal and dermal layers that go through hair cycle processes to produce new hair continuously throughout the life of the organism (9). The HF stem cell (HFSC) is responsible for maintaining tissue homeostasis in response to physiological and pathological conditions. It contains many cell types, including intradermal adipocytes (10), dermal fibroblasts (9), cutaneous blood vessels (10), macrophages, and endothelial cells (11). Immune cells in the HF epithelium constitute the HF immune system (12). Macrophages and T lymphocytes are cutaneous immune cells. Dermal papilla (DP) provides signals that control HF development and contribute to determining the hair shaft's size, shape, and pigmentation (13,14) as well as acting as a reservoir of stem cells (15). An altered DP microenvironment can lead to human skin hair loss, such as AGA or chemically induced alopecia (16–18). The hair cycle is divided into three phases: growth and regeneration, regression, and resting phase (anagen, catagen, and telogen, respectively). Macrophages are linked to HF cycle regulation, particularly in anagen–catagen transition (12,19). During the catagen phase, macrophages digest the excess extracellular matrix and activate the HFSCs, thereby stimulating the HF to enter the anagen phase (20). Macrophages

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entering the anagen phase perform collagen phagocytosis to remodel the matrix (21).

Hair cycle and regeneration are tissue-restructuring processes that include growth factors, cytokines, hormones, adhesion molecules, and related enzymes (22). Growth factors, such as insulin-like growth factor (IGF-1), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), are essential in HF morphogenesis by acting on stem cells in the bulge area of HFs (23,24). Although growth factors have positive effects on HF regeneration, their high cost limits their use in clinical applications (25). Using stem cells and growth factors in cell-based therapies is an accepted therapeutic strategy for damaged-tissue repair because of their direct cellular effects (26). However, human amnion-derived stem cells (hADSCs), human amniotic epithelial stem cells (hAESCs), and human amniotic mesenchymal stem cells (hAMSCs) have greater advantages, including regeneration and tumorigenicity former, than other stem cells. Moreover, acquiring embryonic stem cells from an adult source has no ethical provisions or legal restrictions (27,28). Their high proliferation capacity, multipotency, and immunomodulatory effects also make them a promising source of stem cells for cell therapy in various diseases (29–33). As medical wastes, amniotic fluid stem cells (AFSCs) can be obtained *via* amniocentesis during the pregnancy intermediate stage or cesarean section (33). Although the ability of AFSCs to multidifferentiate is limited, they do not pose a risk for teratoma formation (34).

Amniotic fluid (AF) is a mixed biological fluid containing protein, lipid, carbohydrates, enzymes, urea, electrolytes, hormones, growth factors (IGF-1, PDGF, interleukin [IL]-8, IL-6, transforming growth factor [TGF]-β, tumor necrosis factor alpha [TNF-α], vascular endothelial growth factor [VEGF], and epidermal growth factor [EGF]), stem cell, and stem cell exosomes (35–43). AF and AF-derived mesenchymal stem cells (AF-MSCs), which are important for normal wound healing, can trigger cell proliferation, differentiation, angiogenesis, and chemotaxis, and these processes are necessary for new HF growth (44). AFSC and its cytokines, especially macrophages involved in HF mesenchyme, have interesting regulatory properties in HF homeostasis (20,43,44). Disrupting this connection may lead to clinically important forms of immune-mediated alopecia (45–47).

Studies of AF on alopecia have been demonstrated through the stem cell effect of this fluid. In this study, AF was made acellular and pooled, making it more standardized in terms of its contents. The content has been made safe even against viral and pyrionic contamination (48) caused by gamma radiation. Therefore, this research aimed to create an AF with a longer shelf life and a more effective form and investigate its effect on HF development in rats.

Materials and methods

Identification, collection, and preparation of amniotic fluid forms

We collected human AF (hAF) samples from 10 different donors who had previously approved AF collection during cesarean section in the operating room of Acibadem Mehmet Ali Aydınlar University Atakent Hospital. These samples were then transferred to the laboratory, maintaining at 4 °C. Pooling the AF, which has low immunogenicity, from different donors further reduces the immunogenicity. Generally, relevant examination is recommended to be conducted before collecting hAF from healthy pregnant women, to avoid collecting AF from patients with metabolic diseases. Researchers must also ensure that the hAF is free from viral infections, such as hepatitis B and pathogenic bacterial infection (48,49). Approximately 2 to 5 ml of hAF was obtained *via* amniocentesis in the second trimester of pregnancy. The AF was first passed through a 0.45 µL filter and then a 0.22 µL filter in a laminar airflow cabin. An equal amount of pool was created by melting (Figure 1).

Preparation of amniotic fluid forms

Freezing amniotic fluid (FAF)

The prepared hAF samples described above were dissolved in 2 and 5 ml vials and then stored at >80 °C until the experiment. These samples represented the non-irradiated pool samples (Figure 4).

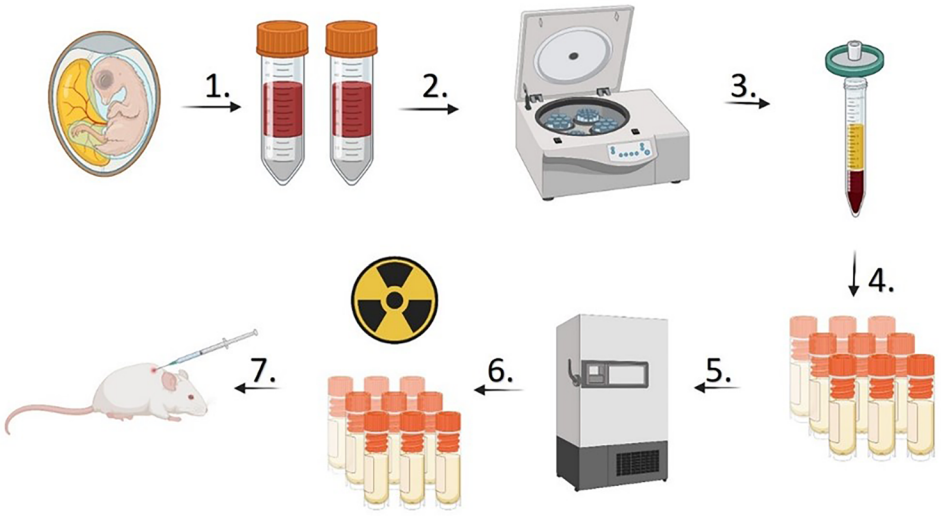

Figure 1. Schematic representation of hAF preparation. (1) Collection of amniotic fluid from at least three different sources. (2) Centrifugation of amniotic fluid to remove macroparticles. (3) Staged filtration of centrifuged amniotic fluid. (4) Pooling of amniotic fluids from different sources together. (5) Freezing of the amniotic fluid at −80 °C to −40 °C. (6) Irradiation in frozen form between 25 and 35 kgy. (7) Subcutaneous application of hAF. hAF, human amniotic fluid.

Freezing gamma-irradiated amniotic fluid (FAFI)

FAF was exposed to 25 Kgray gamma radiation (irradiated FAF) in dry ice at −80°C. Before use, the contents of the vial were resuspended with 1 ml of sterile double-distilled water and used within 2 h.

In vivo animal and experimental design of the hair regeneration model

Albino female Wistar rats (6–8 weeks, 250–300 g) were obtained with follow-up with Acibadem Mehmet Ali Aydinlar University DEHAM (2019-32). The rats were anesthetized with 3% pentobarbital sodium (30 mg/kg), and dorsal skin hairs in rats were removed by mechanical depilation. In the experiment, we randomly assigned the rats to three main groups ($n = 7$/group): (1) Cnt group, treated with normal saline; (2) FAF-treated group; and (3) FAFI-treated group (1 ml). Each treatment was done subcutaneously on days 1, 3, and 5. Rats were kept in a controlled temperature, a 12 h light/dark cycle, and a standard feeding in the nest. We considered all probable side effects, including redness, swelling, and rash, during the experiment period. Animals were anesthetized by subcutaneously injecting ketamine (100 mg/kg) and xylazine (5 mg/kg). We then sacrificed the rats and obtained skin tissues on day 21. Photos were taken for 21 days to record for macroscopic analyses. Furthermore, horizontal biopsies were taken from the center of treatment areas. Anesthesia and sacrifice were in accordance with the standard method. For histological analysis, we collected dorsal skin samples from each animal and examined them in a blinded fashion.

Amniotic fluid analysis

Cytokine ELISA assay in AF

Signosis' Human Growth Factor ELISA Strip II Protein Standards Cat# EA-1102) was used for profiling and measuring human growth factors (VEGF, EGF, PDGF-B, TGF-B, FGF-B, IL-1, and IL-6). In accordance with the manufacturer's protocol, we added 200 µL of diluent buffer to the wells of the first strip, and 100 µL to those of the remaining strips. The results were expressed as ng/L.

Cytokine bead array (CBA) from AF:Th1/Th2 cytokine ratios

The ratio of cytokines to diluted AF was 1:2. Such cytokines included TNF-α/IL-10, TNF-α/IL-6, IFN-ɣ/IL-6, interferon alpha (IFN-α)/IL-6, granulocyte-macrophage stimulating factor (GMCSF)/IL-6, GMCSF/IL-10, and GMCSF/IL-9. These ratios were evaluated by CBA (Miltenyi Biotec MACSPlex Cytokine 12 Kit, human; cat #130-099-169) according to the manufacturer's protocol. The experiment was repeated thrice. In addition, flow analysis was conducted at Beckman Coulter.

AF-induced macrophage differentiation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood collected in a citrate tube through the Ficoll gradient method (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). We inoculated 500.000 PBMCs/well in 24-well plates. After 2 h of incubation, we discharged the suspended cells and observed the adherent monocytes. Dilutions were prepared with Dulbecco's Modified Eagle Medium to achieve final AF concentrations of 0%, 5%, 10%, and 20%. After 24 h of incubation, the adherent monocytes were collected with trypsin and prepared for flow cytometry analysis (CD14-APC [cat#982506], CD86-Percpcy7 [cat#374209], and CD206-FITC [cat#141703]). Moreover, flow analysis (Beckman Coulter) and Student's *t*-test analyses were conducted.

Nanoparticle tracking analysis (NTA)

The number of microvesicles in AF was determined using Te NANOSIGHT NS300 (Amesbury, UK). Samples were diluted with distilled water at a 1:10 ratio and transferred to NANOSIGHT cuvette at 1 ml. Measurements were conducted at room temperature with five different 60 s video recordings and repeated thrice.

Histopathological sampling analyses

Histochemical and immunohistochemical (IHC) stainings

Skin samples were fixed in 10% buffered neutral formalin for 48 h. The detected tissue samples were dehydrated by passing through an alcohol series according to the histological tissue follow-up method, and after clearing in xylol, they were embedded in paraffin. Then, serial sections at 5 µm thick were prepared (50).

Hematoxylin–Eosin (H&E) staining

HF parameters were stained with H&E (H3136 Sigma-Aldrich Hematoxylin and E4009 Sigma-Aldrich Eosin Y). The thickness of the dermis (the skin's adipocyte layer) and the number of HFs and anagen phases were counted in three areas (9 sections) from a biopsy on three random fields in one section using a high-power microscope field by a histologist who was blinded to the codes.

Masson's trichrome staining

To observe collagen fibers, we stained them with Masson's trichrome staining (HT15-1KT, Sigma) kit and evaluated them in light microscopic images.

IHC staining

Sections of 5 µm thickness obtained from the selected paraffin blocks were secured in poly-L-lysine–coated slides and kept in an oven at 37°C for one night. Afterward, deparaffinized sections were placed in xylene for 10 min and dehydrated by keeping them in 96% pure alcohol for 5 min. Each section was then washed in distilled water for 2 min. Thereafter, to ensure antigen retrieval, we heated it in a citrated buffer (pH 6) solution at 98°C for 20 min and cooled it at room temperature for 20 min in the same buffer. Afterward, IHC staining was started. First, to eliminate endogenous peroxidase activity, each skin section was blocked with 3% hydrogen peroxide, incubated for 20 min, and washed with phosphate-buffered saline solution (PBS) for 5 min. Subsequently, the protein block (Large Volume Ultra V Block, TA-125-UB®, Lab Vision Corporation, Fremont, CA, USA) was applied for 5 min. Before washing the sections, we shook the blocking solution and applied the primary antibodies arginase 1 (Arg1; Biocare, cat#ACI3058) and CD68 (Dako cat#M0814). Thereafter, primary antibodies were washed in PBS for 5 min, and secondary antibodies were dripped and incubated for 20 min. After washing for 5 min in PBS again, the tertiary antibody was dropped and incubated for 20 min. The 5 min PBS wash was repeated. Afterward, chromogen was added to diaminobenzidine, incubated for 5–15 min, and washed in distilled water. Tissues were counterstained in Mayer's Hematoxylin solution for 1 min, washed in distilled water for 2–5 min, and passed through an alcohol. The air-dried preparations were placed in xylene, covered with Entellan (LB.M.107961.0500, Merck), and used for the specified measurements.

Quantitative histomorphometry

Individual HFs were shown in the photomicrographs of H&E-stained longitudinal sections of every rat. We counted photomicrographs

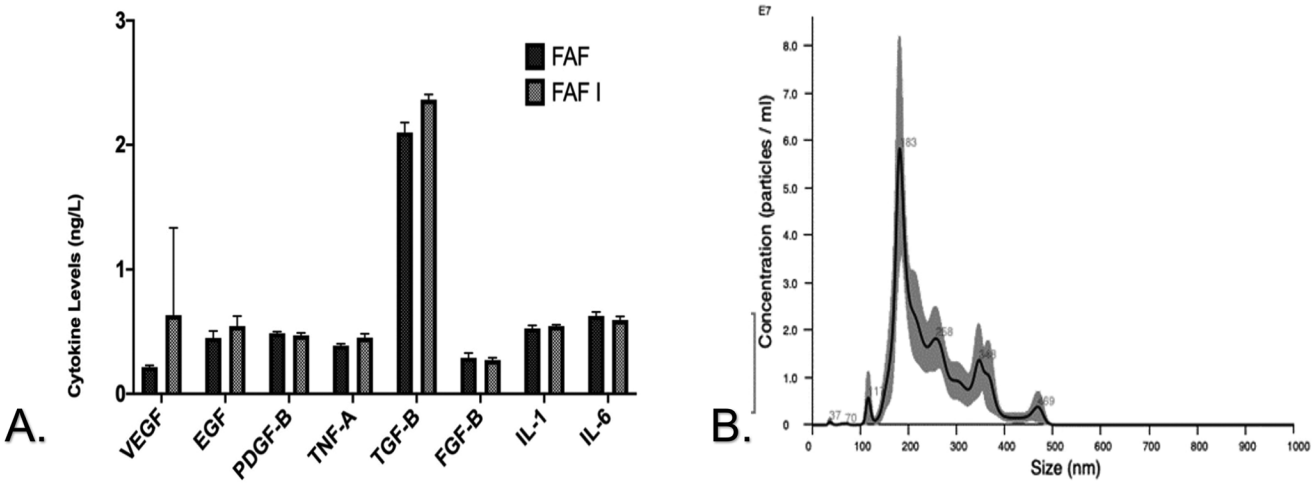

Figure 2. Statistical comparison of the levels of cytokines (VEGF, EGF, PDGF-B, TNF-α, TGF-β, FGB-B, IL-1, and IL-6) in FAF and FAFI groups in an experimental hair follicle development model (ng/L) (a). Our data show the transition of M1 to M2 in hAF-treated groups and the high exosome content (1.14e10 ± 0.531e10) of the amniotic fluid (b). FAF, frozen amniotic fluid: FAFI; FAFI, frozen irradiated amniotic fluid: FAFI; hAF, human amniotic fluid.

in the same region (1300 m wide) of the HFs. In each group, the percentage of HFs in a particular anagen phase was calculated.

Cell counting of Arg + 1/CD68-polarized macrophages in in vivo skin analogs

In the sections, the stained CD68 antibody was evaluated as the macrophage, and the stained Arg + 1 antibody was evaluated as M2 rat macrophages of the cell density. In nine different sections of each skin analog, three were randomly selected and counted at 10× high-power fields. Cells showing distinct immunopositive reactions for CD68 and Arg + 1 were counted per 0.2 mm^2 from randomly selected areas in the FAF, FAFI, and Cnt groups. Immune cell density was expressed using the average total number of CD68- or Arg + 1-positive cells quantified per high-power field of a skin section. Results are presented as the mean and standard deviation (SD).

Statistical analysis

The outcomes are shown as the mean and SD. One-way analysis of variance (ANOVA) was used for data analysis. All statistical data were analyzed using SPSS version 23, and a *p*-value of 0.05 was considered significant.

Results

Amniotic fluid analysis results

As shown in Figure 2(a), TGF-β ($p < 0.00714$) and VEGF ($p < 0.00003$) levels were higher in the FAFI group than in the FAF group. When Th1-type cytokines were divided by the amounts of Th2-type cytokines, the values were below 1 (Figure 2b).

Macrophage 2 profiles in amniotic fluid and tissue

Th2 cytokines were linked to changes in the expression levels of these cytokines (Figure 3a). The Th2 type cytokines of AF at 10% concentration or more can induce switching of the M1 phenotype to M2 phenotype in macrophages (Figure 3b). The M2 macrophage marker Arg + 1 cells were expressed around DP (black arrow) and stroma (red arrowhead) in the samples (A: FAFI; B: FAF; C: Cnt). The CD68 +cells were also expressed around DP (black arrowhead) in all samples (D: FAFI; E: FAF; F: Cnt) (Figure 3c). Furthermore, the rate of Arg + 1/CD68 cells found in the FAF and FAFI specimens significantly increased compared with that in the Cnt group ($^*p < 0.05$). All data are reported as the mean ± SD (Figure 3d).

Macroscopic and microscopic analysis results

Effects of the FAF and FAFI on the transition to anagen phase in rats

Figure 4(a–c) shows the HF patterns in each group during macroscopic and histological analyses at a vertical view from the dorsal portion of rats. The transition of HFs to the anagen phase was accelerated (Figure 4b–d) in the hAF-administered groups (FAF and FAFI) compared with that in the Cnt group (Figure 4d). At day 21, the HFs in the Cnt group were in the early anagen phase, appearing as hair bulbs in the dermis. In the FAF and FAFI groups, the HFs were in the late anagen phase, displaying the largest hair bulb size, the deepest HF in the subcutis, and the newly formed hair shaft reaching the level directly below the sebaceous gland.

Effects of the FAF and FAFI on hair growth in rats

Figure 4(e–f) illustrates the histological evaluation of HFs and collagen fibers using H&E and Masson staining, respectively. In this model, the FAF and FAFI groups had more regenerated HFs than the Cnt group (Figure 4e). Especially, collagen fibers were less accumulated and more neatly arranged in these hAF-treated groups than in the Cnt group (Figure 4f). In the representative longitudinal sections, the number of HFs was significantly increased in the FAFI and FAF groups compared with that in the Cnt group ($p < 0.001$ and $p < 0.0008$, respectively) (Figure 4g). In addition, the number of HFs was significantly greater in the FAFI group than in the FAF group (Figure 4g).

Effects of the FAF and FAFI on adipocyte and dermal layers in rats

Figure 4(h) depicts the histological evaluation of the thickness of the dermal and adipocyte layers using H&E staining. We quantified dermal and adipocyte layer thickness in dorsal rat skin on day 21

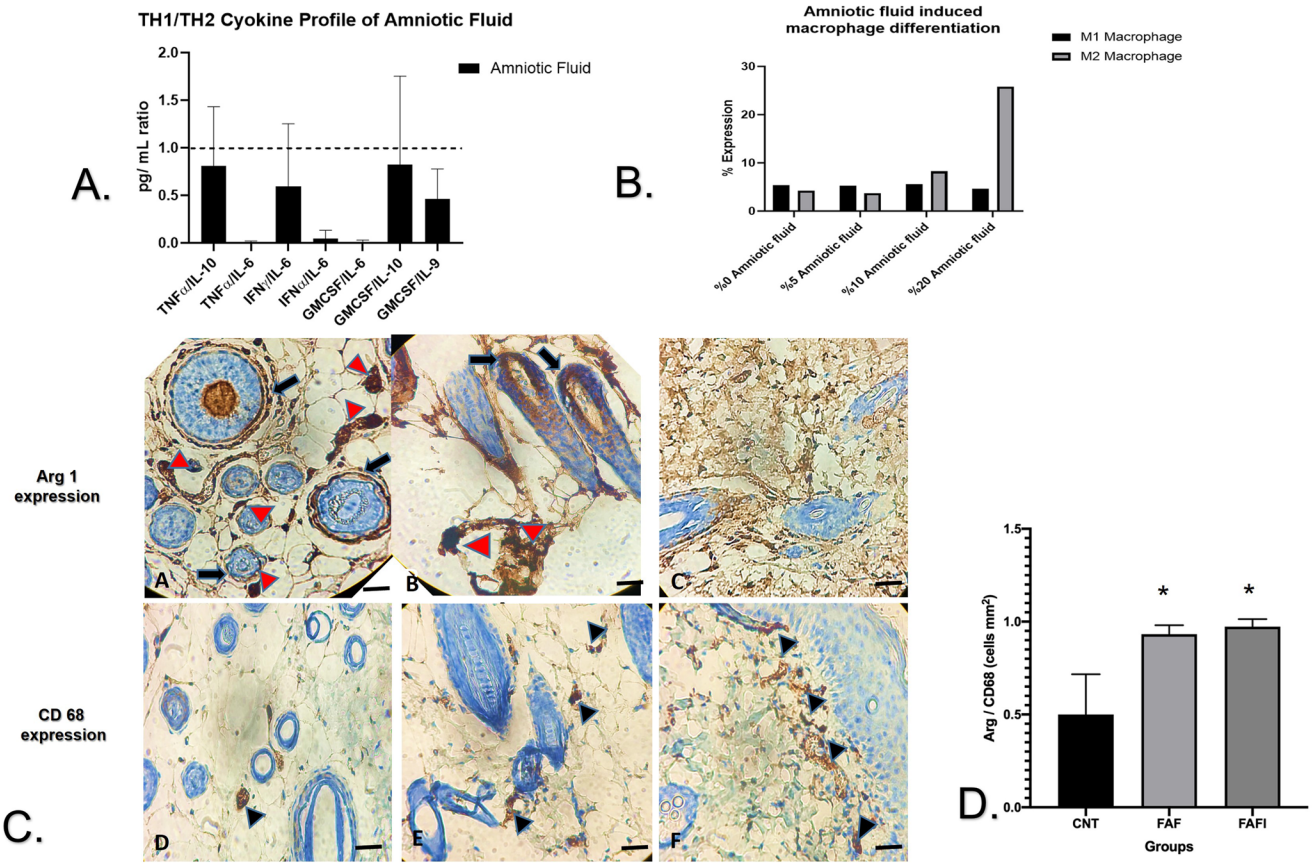

Figure 3. Differential hAF expression with macrophage differentiation (a). Th2-type cytokines found in the amniotic fluid (b). Macrophage cell counts in skin specimens (c). Arg + and CD68+ cell counts (cells per mm^2 specimen area) in the whole analyzed area in the stroma compartment of the skin specimens (d). ANOVA test's p-value results are shown. Scale bars, 400 µm. hAF, human amniotic fluid.

(Figure 4i–j). The dermal layer did not change in all groups ($p > 0.005$) (Figure 4i), but the adipocyte layer thickened during HF morphogenesis in the FAFI and FAF groups ($p < 0.005$) (Figure 4j).

Discussion

The study is the first to investigate the ability of hAF to interact with various immune cells residing within the skin to promote hair growth and to compare the effectiveness of two types of hAF, FAF and FAFI, in stimulating hair growth. Stem cell of hAF has already been known to be involved in HF regeneration (42,51–54). We focus on the immune system of the skin, specifically around the hair follicle. Mast cells, macrophages, Langerhans cells (LCs), and T cells tend to preferentially associate with the hair follicle (HF) in both murine and human skin. This specialized immune cell clustering around the hair follicle plays a significant role in various skin processes, including inflammation and immune responses (51,55). Xiao et al. stated that an altered skin immune microenvironment is associated with various dermatological disorders, such as pathological alopecia areata (56). Numerous studies have demonstrated that T cells and macrophages serve as key immune regulators in hair follicle regeneration during normal hair cycling (44,57) and in hair neogenesis following injury (58). Notably, the composition of immune cells in the perifollicular space differs from that in the surrounding interfollicular space. Understanding the precise mechanisms by which immune cells contribute to alopecia is essential for developing more effective and targeted therapies for this condition. In this study, we injected FAF and FAFI into rat and observed the transition from M1 to M2 macrophages was associated with hair follicle regrowth. In our study, M1 activation, as indicated by CD68 expression, was not observed in the rats treated with FAF and FAFI (59,60). This finding suggests that FAF and FAFI may promote a favorable immune environment for hair follicle regeneration by suppressing M1 activation and facilitating the transition to the M2 phenotype. HF degeneration has been linked to an anagen-related increase in macrophage numbers (22,26,44,61). In our study, we also observed that FAF and FAFI led to an increase in M2 macrophages during the anagen phase, followed by a significant decrease during the catagen phase and a further reduction to minimal levels during the telogen phase (51,62). Previously, notable fluctuations in immune cell numbers have been shown to correlate with hair cycling (62–66). These results suggest that macrophages play a crucial role in the regulation of hair follicle cycling, with their activity fluctuating in response to different hair cycle stages. Furthermore, significant anagen transition and hair growth were observed in the FAFI group, highlighting its enhanced effectiveness. These findings raise the question of why the irradiated group (FAFI) is more effective than the non-irradiated group (FAF). Previous research has suggested that gamma irradiation can stabilize growth factors by inhibiting protease activity, which would otherwise degrade active substances in a liquid (48). This protective effect of gamma irradiation may explain the enhanced efficacy observed in our study.

The use of growth factors in treating alopecia has shown promising potential for promoting hair growth. Our data demonstrated

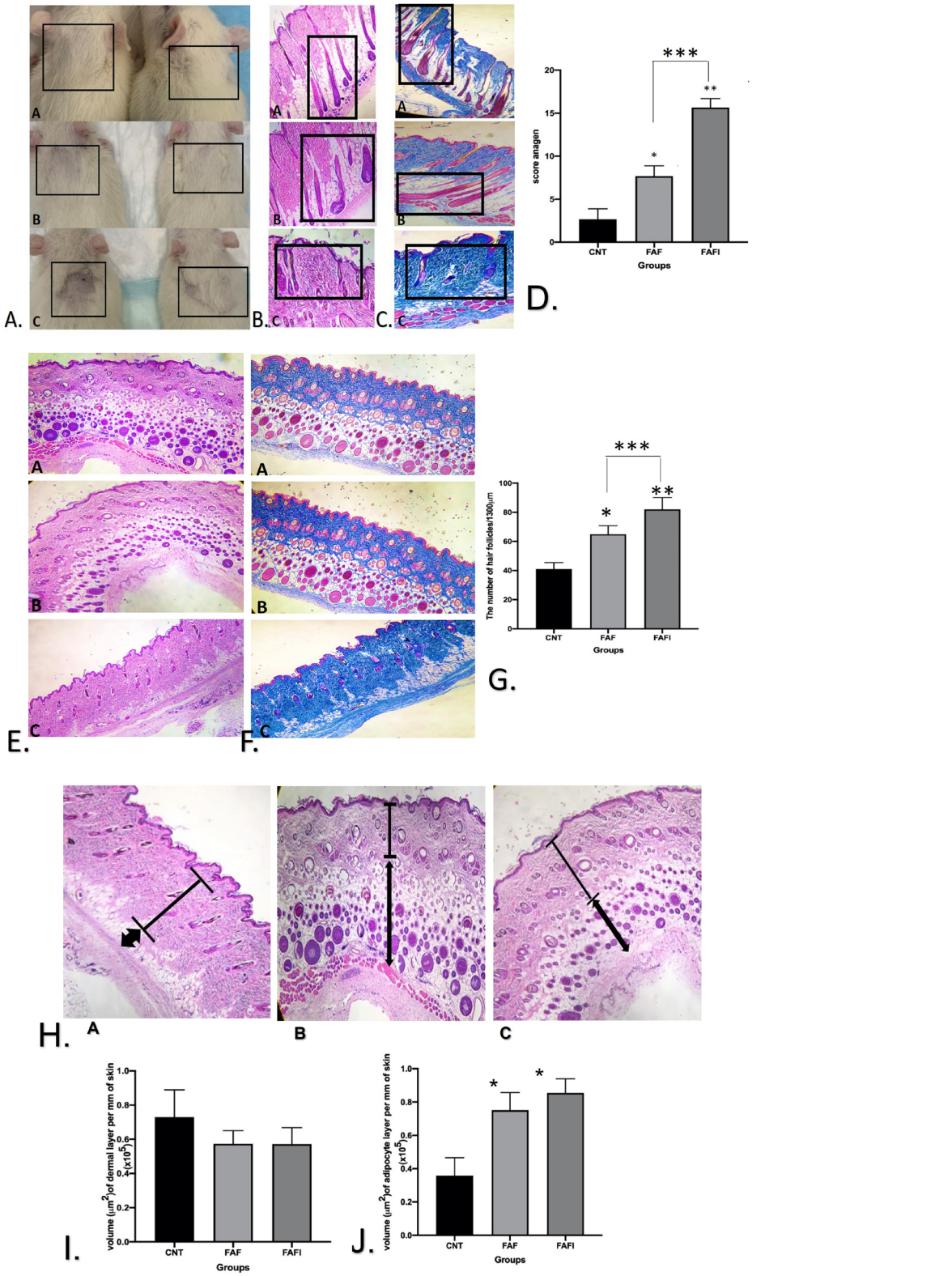

Figure 4. Rat hair follicle development (black box) (a) and the anagen phases in the sections (b, c). Hair follicle development evaluated by H&E staining (b), masson's trichrome staining (c), and anagen scoring (d). Typical photos of dorsal skin (left panel) examined by histological analysis (right panel) (**A:** FAFI **B:** FAF, **C:** Cnt) ×100. Representative photomicrographs of transverse sections stained with H&E (e) and masson's trichrome (f), with hair follicle scoring (g). After 21 days, the hair follicle in the skin was analyzed, and its total number was quantified using histology images obtained by Image J software (c, 10×). (A: FAFI, B: FAF and C: Cnt groups) ×100. *$p < 0.001$; **$p < 0.0008$ compared with the Cnt group. ***$p < 0.04$ compared with the FAF group. The transition of HFs to the anagen phase was quantified using histology images with Image J software (D-10×). *$p < 0.05$; **$p < 0.0001$ compared with control, *** $p < 0.0001$ compared with FAF group. The relationship between dermal layer thickness and dermal adipocyte layer thickness was analyzed; double-headed arrows indicate the adipocyte layer, whereas bars indicate the dermal layer (h). Cnt (a), FAF (B), and FAFI (C) (H&E scale bar, 200 µm). Quantification of the dermal (i) and adipocyte layer thickness (j) in rats. Dermal layer thickness did not differ between all the groups ($p > 0.05$). Adipocyte layer thickness significantly increased in the FAF and FAFI groups compared with that in the Cnt group (*$p < 0.05$) (j). Two-way ANOVA followed by Bonferroni's multiple comparison test. All data are reported as the mean ± SD.

that TGF-β and VEGF levels were significantly elevated in the FAFI group. Similar to our findings, TGF-β has also been shown to increase Arg1 activity (67), and its presence was detected in anagen hair follicles in previous studies (68). This increase was associated with accelerated transition to the anagen phase and enhanced hair follicle (HF) growth. These growth factors are known to play critical roles in activating HF morphogenesis and regulating the hair cycle (69), further supporting their importance in hair regeneration have shown promise in reversing hair loss in alopecia areata.

Furthermore, phagocytosis of cellular debris by M1 macrophages can promote the production of TGF-β, one of the Th2 cytokines, while reducing the expression of the Th1 cytokine TNF-α, reflecting their transition to the M2 phenotype (70). In our study, it was also observed that the irradiated amniotic fluid showed higher levels of TGF-β and lower levels of TNF-α. In particular, the M1/M2 balance is critical in determining the balance between fibrosis, inflammation/regeneration damage, together with the cytokine microenvironment. M2 polarization can be obtained *in vitro* with M-CSF, IL-4, IL-6, IL-10, IL-13, IL-33, and/or TGF-β and Arg + 1 (71–80). M1 macrophages release pro-inflammatory cytokines and engulf dead cells and pathogens. Under the influence of bacterial metabolites, IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF), they can adopt this phenotype (81). As demonstrated in our study, macrophages likely play a crucial role in maintaining this immune balance, as cytokines such as GM-CSF and IL-10 contribute to the regulation of macrophage activation and the modulation of inflammation. According to the concept of M1/M2 polarization, IFN-γ, TNF-α, and IL-1β are possible factors contributing to the activation of M1 macrophages (82). M1/M2 macrophages functionally correspond to Th1 and Th2, respectively (83). Th2 cytokines can increase the ornithine and urea production of Arg + 1, which, in turn, increases macrophage arginine metabolism (84,85). In our study, the cytokine ratios being below 1 in the amniotic fluid may indicate that a regulated anti-inflammatory response is being mediated through macrophages, with a reduced risk of excessive inflammation. Additionally, we found that AF concentration of 10% or more was found to be related to the M1-to-M2 phenotype switch in macrophages. In addition, In parallel with our study, Tan et al. reported the conversion of M1 into M2 in amnion epithelial cell content (86,87). AFSCs provide protection by modulating immune function, particularly through the regulation of macrophage recruitment and phenotype, including the conversion of M1 macrophages into M2 macrophages (88–91). However, whether M1 is involved in excessive hair shedding is unclear and more studies should be conducted.

Another factor that enabled the transition of M1 to M2 in hAF-treated groups was that the AF contained high exosome content (1.14e10 ± 0.531e10). Indeed, a previous study showed that hAFSC exosomes contain HGF and TGF-β through the extracellular vesicles secreted by AFSCs (92) Furthermore, hAFSC exosome treatment improved the regeneration levels of HFs, nerves, and vessels (54).

Preadipocyte proliferation can be stimulated through angiogenesis (11) or cytokines such as PDGF, EGF, IGF1, and FGF (10,98,99) which activate DP cells to promote the hair growth cycle and transition to anagen phase (9,10). We found that the adipocyte layer thickened in the hAF-injected groups. In our study, as well as in previous findings (9,10,92–100), it was observed that the thickness of the dermal adipocyte layer increased during the anagen phase, but decreased during the catagen and telogen phases. Immature dermal adipocytes also activate HF stem cells to initiate the hair growth cycle (19).

Our current histological analysis demonstrates that irradiated hAF accelerates HF regeneration and reduces collagen fiber deposition. This suggests that irradiated hAF not only promotes the regeneration of hair follicles but may also play a role in modulating the extracellular matrix, potentially preventing excessive fibrosis during the hair growth cycle. This finding highlights the therapeutic potential of irradiated hAF in hair restoration and tissue remodeling.

In summary, the exosome content of amniotic fluid (AF) and the contribution of growth factors facilitated the conversion of M1 to M2 macrophages during hair regeneration. This process stimulated M2 polarization and led to an increase in the dermal adipocyte layer, which, in turn, promoted hair follicle (HF) development. Furthermore, gamma irradiation enhanced the effectiveness of the AF product, possibly through protease inactivation, while also ensuring its safety. Therefore, pooled and irradiated hAF may offer a promising therapeutic option for the clinical treatment of alopecia and in addressing the pathobiology of HF growth disorders. These findings will be further evaluated in an upcoming clinical trial.

The focus on irradiated hAF, which is not only safe and easily accessible but also does not raise ethical concerns when applied in the clinical setting, holds great promise. It could enrich not only translational hair research but also serve as a model for other research fields. Thus, our study provides evidence that hAF supports HF regeneration through cytokines, M2 macrophages, and extracellular vesicles. These characteristics position hAF as a valuable source of FAFI for cell therapy and regenerative medicine.

Conclusion

As an alternative to cell therapy, hAF, especially gamma-irradiated pooled hAF, may be beneficial in HF-regenerative treatments in clinical settings.

Patents

National (2022/009965) and international (PCT/TR2022/050661) patent numbers of this method have been received.

Acknowledgments

In this section, you can acknowledge any support given that is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g. materials used for experiments).

Authors' contributions

Conceptualization, GTT. and EO.; methodology, GTT; EO.; software, GTT; EO.; validation, GTT; EO.; formal analysis, GTT. and EO.; investigation, GTT; BY; EG; DC.; resources, GTT; data curation, GTT; EO writing—original draft preparation, GTT.; writing—review and editing, GTT. and EO.; visualization, GTT. and EO.; supervision, GTT. and EO.; project administration GTT. and EO.; funding acquisition, EO.

Disclosure statement

The authors declare no conflict of interest.

Institutional review board statement

The ethical approval of this study was authorized by the Acibadem Mehmet Ali Aydinlar University Local Ethics Committee for Animal Experiments (ACU-HADYEK) with the decision number 2019/32 on the 12th of March 2019. All procedures in this study were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

Funding

This research received no external funding.

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