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Fabrication of Cell-Laden AME-Loaded Collagen-Based Hydrogel Promotes Fibroblast Proliferation and Wound Healing *In Vitro*

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Abstract

Objective: The biological factors secreted from cells and cell-based products stimulate growth, proliferation, and migration of the cells in their microenvironment, and play vital roles in promoting wound healing. The amniotic membrane extract (AME), which is rich in growth factors (GFs), can be loaded into a cell-laden hydrogel and released to a wound site to promote the healing of the wound. The present study was conducted to optimize the concentration of the loaded AME that induces secretion of GFs and structural collagen protein from cell-laden AME-loaded collagen-based hydrogels, to promote wound healing *in vitro*.

Materials and Methods: In this experimental study, fibroblast-laden collagen-based hydrogel loaded with different concentrations of AME (0.1, 0.5, 1, and 1.5 mg/mL, as test groups) and without AME (as control group), were incubated for 7 days. The total proteins secreted by the cells from the cell-laden hydrogel loaded with different concentrations of AME were collected and the levels of GFs and type I collagen were assessed using ELISA method. Cell proliferation and scratch assay were done to evaluate the function of the construct.

Results: The results of ELISA showed that the concentrations of GFs in the conditioned medium (CM) secreted from the cell-laden AME-loaded hydrogel were significantly higher than those secreted by only the fibroblast group. Interestingly, the metabolic activity of fibroblasts and the ability of the cells to migrate in scratch assay significantly increased in the CM3-treated fibroblast culture compared to other groups. The concentrations of the cells and the AME for preparation of CM3 group were 10^6 cell/mL and 1 mg/mL, respectively.

Conclusion: We showed that 1 mg/ml of AME loaded in fibroblast-laden collagen hydrogel significantly enhanced the secretion of EGF, KGF, VEGF, HGF, and type I collagen. The CM3 secreted from the cell-laden AME-loaded hydrogel promoted proliferation and scratch area reduction *in vitro*.

Keywords: Amniotic Membrane Extract, Fibroblast, Growth Factor, Hydrogel, Wound Healing

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Introduction

Wound healing is a complex biological process that requires the successful completion of different healing stages, namely homeostasis, inflammation, proliferation, and regeneration (1). During the wound healing process, cytokines and growth factors (GFs) are the vital biological factors that stimulate the synthesis of DNA and cell mitosis (2). In addition, they can regulate various cellular functions, such as proliferation, migration, differentiation, morphogenesis, and apoptosis, which accelerate wound healing (3). Recently, the action mechanisms of several GFs have been revealed in this process, and some of them, including epidermal GF (EGF), hepatocyte GF (HGF), keratinocyte GF (KGF), platelet-derived GF (PDGF), transforming GF beta (TGF-b), vessel endothelial GF (VEGF), fibroblast GFs (FGF), and insulin GF (IGF), are commercially available for both clinical use and research (4). Among them EGF, HGF, FGF, and KGF stimulate the proliferation of fibroblast, keratinocyte, and vascular endothelial cells by different mechanisms (5). VEGF

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affects angiogenesis and granulation tissue formation in the early stages of healing (6), while PDGF is vital for inflammation, granulation, and epithelial regeneration through wound healing stages (2, 7). Although the roles of different GFs during healing process have been studied by different researchers, all monotherapies based on the GFs have failed (8).

Today, using cell-based products, such as cell-laden skin substitutes on the wound bed, remain a promising strategy in skin regeneration (9). Skin substitutes have been introduced to the wound care market since 1975 (10). They can modulate the physicochemical and biological features of the wound environment through different molecular mechanisms including interactions among GFs, cells, and extracellular matrix (ECM) (11). Fibroblasts are known as important cells for skin wound reconstruction and repair through skin substitute implantation (12). They can produce a suitable concentration and combination of cytokines, and provide ECM components that promote wound healing (13, 14).

The amniotic membrane (AM) as a naturally-occurring biologically-active construct has been used both as wound dressing and tissue replacement for different types of wounds during recent years (15, 16). It contains various GFs such as PDGF, KGF, HGF, bFGF, EGF, and VEGF, and also glycosaminoglycans (GAGs) for example, hyaluronic acid in its construct. Therefore, it can increase the proliferation of human skin fibroblasts and also mesenchymal stem cells (17-20). A variety of research have revealed the importance of GAGs and their analogues in acceleration of skin cell migration (21, 22). In addition to being a source of GFs, the AM has the potentials to act as a highly bioactive and natural scaffold for the growth, migration, and adhesion of keratinocytes and fibroblasts, resulting in wide-ranging applications in skin tissue engineering (19). Furthermore, AM reduces the risk of infection, owing to its antimicrobial properties, which not only acts as a biological barrier, but also expresses several antimicrobial molecules such as beta3-defensin and elafin (23). Additionally, human amniotic epithelial cells (HAECs) produce high levels of cytokines, which are largely effective in wound healing for acute and chronic wound models (24).

Recently, we developed an extract from AM called AM extract (AME), and evaluated its role on skin wound epithelialization (25, 26) and corneal damage (27). Based on our previous studies, we assumed that the biological behaviors of fibroblasts may be affected by some specific concentrations of AME. Therefore, the main aim of this study is to evaluate the effects of different concentrations of AME loaded in hydrogels on GFs and structural proteins that release from cell-laden collagen-based hydrogels and induce fibroblast proliferation and accelerate wound healing *in vitro*.

Materials and Methods

In this experimental study, human amniotic membranes (HAMs) were obtained from Royan Stem Cell Technology (Iran). All HAMs were negative for microbial, fungal, and viral contamination. Dulbecco's Modified Eagle Medium/F12 (DMEM/F12), fetal bovine serum (FBS), and fungizone were obtained from Gibco. Trypsin, ethylenediaminetetraacetic acid (EDTA), penicillin/streptomycin, acetic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and L-glutamine were supplied by Sigma-Aldrich. Sodium hydroxide (NaOH) and trypan blue were purchased from Merck. Mitomycin C (MMC) (ab120797) was purchased from Abcam. Rat tail collagen and human dermal fibroblasts (HDFs) were obtained from Royan Institute and Stem Cell Bank of the Pasteur Institute, respectively.

Preparation of amniotic membrane extract

The AME was prepared according to our previous study. Briefly, The HAMs were cut into small pieces, were immersed in liquid nitrogen and then pulverized with a grinder machine (SC-7880 Silver Crest). The prepared powder was dispersed in DMEM/F12 medium and sonicated (UP200S-Heilescher) three times at low temperature (0°C) for a maximum power of 20% of the working cycle for 10 minutes. The homogenate was centrifuged at 4000 g for 10 minutes, then 15,000 g for 5 minutes. Finally, the supernatant was filtered through a 0.2 μm filter, aliquoted and kept frozen at -70°C until use (27).

Fibroblast cell culture

HDF cells in passages 5-6 were cultured in DMEM/F12 medium with FBS (10%), penicillin-streptomycin (100 units/mL), and fungizone (0.25 μg/mL) (28). Culture flasks were incubated at 37°C, with 5% CO_2 concentration and 95% relative humidity. Once the cells reached 80% confluence, fibroblasts were removed from the culture flasks with trypsin/EDTA (0.1% w/v, 0.02% w/v) and transferred into T-25 flasks. This study was approved by the Royan Institute Ethics Committee (IR.ACECR.ROYAN.REC.1398.157).

Preparation of cell-laden amniotic membrane extract-loaded collagen hydrogel

Fibroblasts were counted by trypan blue and then encapsulated in a physically-crosslinked collagen hydrogel (0.6 mg/mL). To this end, a stock solution of type I collagen (obtained from rat tail) at a concentration of 1.2 mg/mL was dissolved in acetic acid (0.1 % v/v). Cell-laden collagen hydrogels were prepared through mixing of the collagen solution (1 mL), DMEM/

F12 supplemented with 10% FBS (1.1 mL), NaOH aqueous solution (0.1 mL, 0.1 M), the cell suspension (0.20 mL, 1×10^6 cells/mL) and varying concentrations of AME (0.1, 0.5, 1, and 1.5 mg/mL) at 0°C. The resulting mixture was poured into a mold and stored at 37°C to form a hydrogel structure. The prepared hydrogels, which were in a disc shape (diameter of 10 mm and thickness of 1 mm, Fig.1), were transferred to a Petri dish containing cultured medium, and were incubated in DMEM/F12 (500 µL) at 37°C, 5% CO_2 and 95% relative humidity for We selected day 7 based on our previous research (22) and other similar reports (8, 29, 30). Moreover, performing a pilot study at different timepoints (1, 3, 7 and 14 days) post-incubation confirmed that day 7 is the optimal time for performing a total protein assay (data not shown). The medium containing total proteins released from the constructs in each group (Table 1) was referred to as conditioned medium (CM) and was stored at -80°C.

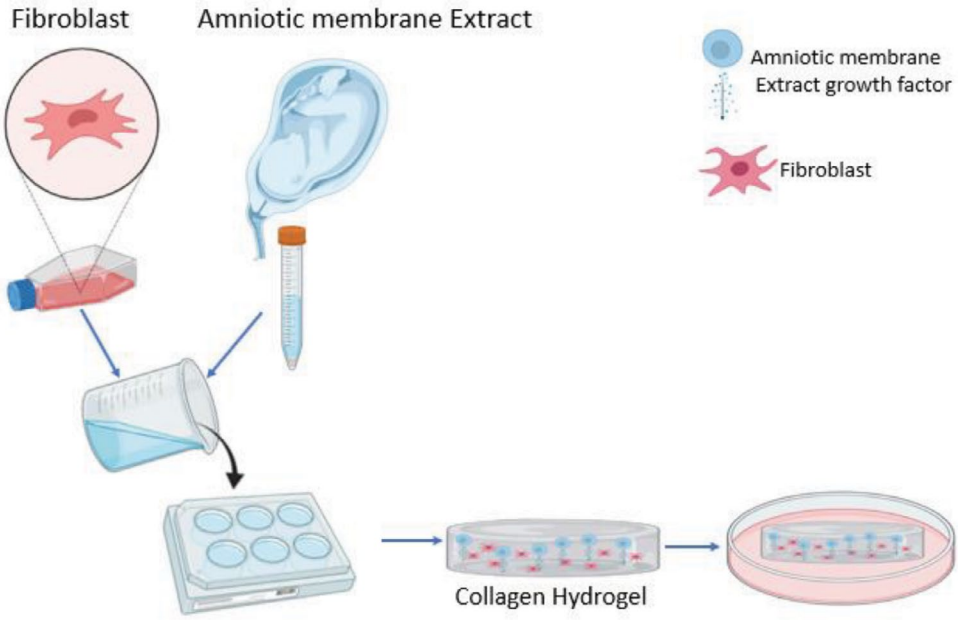

Fig.1: Schematic representation for preparation of cell-laden, amniotic membrane extract (AME)-loaded collagen hydrogel. The AME and fibroblasts were combined with collagen solution and poured into a mold. The resulting hydrogel was placed in culture medium, and the subsequent conditioned medium was removed for analysis after days 7.

Table 1: Different experimental groups to assess their efficacy on GFs secreted from hydrogels

Group	Ingredients	AME concentration in hydrogel (mg/mL)	Fibroblast (cells/mL)
AME	Only AME	1	-
CM1	AME+FIB	0.1	1×10^6
CM2	AME+FIB	0.5	1×10^6
CM3	AME+FIB	1	1×10^6
CM4	AME+FIB	1.5	1×10^6
FIB	FIB	-	1×10^6

CM (1-4); The conditioned medium, which is total proteins secreted by the cell-laden hydrogel that was loaded with different concentrations of AME, AME; Amniotic membrane extract,FIB; Fibroblast, and GFs; Growth factors.

Quantitative analysis of growth factors secreted by the cells from conditioned medium

The total protein levels for AME and CM were evaluated using the bicinchoninic acid (BCA) method. CM samples as the analytes were collected from each group and stored at -80°C. In general, 25 µL of the analyte and serially diluted standards (0.063,,0.125 1.5 ,1 ,0.75 ,0.5 ,0.25, and 2 µg/µL) were added to each 96-wells plate. The plate was covered, incubated at 37°C for 30 minutes, cooled down to room temperature and then, the optical density (OD) of the samples was read in triplicates by a spectrophotometer (Multiskan Spectrum, Thermo Scientific) at 520-570 nm. In this context, cumulative concentrations of the growth factors EGF, KGF, HGF, and VEGF, as well as some important proteins present in AME, such as the structural protein (type I collagen), were evaluated using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems) according to the manufacturers' protocols.

Effect of conditioned medium on fibroblast proliferation

Human fibroblasts were cultured (2.5×10^5/well) in a 6-well plate at 37°C, 5% CO_2 concentration and 95% relative humidity. The cells were seeded and cultured in a culture medium (1 mL) with penicillin G/streptomycin (1%), and L-glutamine (200 mM). The collected CM samples (1 mL) were added to each well. The cells cultured in the presence of FBS (10%) and the basal culture medium (DMEM) were considered as the positive and negative controls, respectively. After 24, 48, and 72 hours of cell seeding, the wells (three wells for each group) were washed with phosphate buffer saline (PBS) and their viability was assessed using the MTT assay. In addition, the cell counting was performed using trypan blue at predetermined timepoints. Each test was repeated three times.

The effect of conditioned medium on fibroblast migration

The effect of each CM sample on *in vitro* cell migration in the fibroblast cultures was evaluated using scratch assay. Briefly, the cells (2.5×10^5/well) were cultured in 6-well plates and during the time that they were incubated at 37°C for 2 hours, proliferation of the cells was inhibited using mitomycin-C (5 µg/mL) (31). After reaching 80% of confluency, the population of the HDFs were scratched with a pipette tip along a straight line. After washing the cell debrides with PBS, the cultured cells were treated with serum-free culture medium containing CM samples. The serum-free, CM-free medium and complete culture medium (DMEM/F12 containing FBS 10%) were selected as negative and positive control samples, respectively. At 0, 12, 24, and 48 hours after scratching, digital images of the cells were taken by an Olympus device and analyzed by Image-J software. Each experiment was repeated three times for every timepoint. The amount of scratch closure (%) was calculated as follows:

$$\text{Amount of scratch closure (\%)}=[(S_0 - S)/S_0]\times100$$

where S_0 is the scratch area at time 0, and S is the scratch area at times 3, 12, 24, and 48 hours.

Statistical analysis

All experiments were performed at least three times, and the results were expressed as mean ± standard deviation (SD). Due to our sample sizes, Kruskal-Wallis Rank Sum Test (non-parametric version of ANOVA test) was used to compare the median of the outcomes across groups. In addition, following a significant Kruskal-Wallis test, Dunn's test was employed for multiple pairwise comparisons along with adjusted P values to account for multiple testing (using Bonferroni approach). A $P<0.05$ was considered significant for all statistical tests. All analyses were performed using R Statistical Software (v4.2.2; R Core Team 2022).

Results

The content of growth factors and structural proteins in prepared amniotic membrane extract

The concentration of GFs and structural proteins in AME is an important parameter that can affect its biological properties. Our results indicated that the concentration of type I collagen and different GFs including EGF, HGF, KGF and VEGF were 341 ± 38.1 µg/mL, 2.3 ± 0.05 µg/mL, 58.1 ± 1.6 µg/mL, 0.28 ± 0.01 µg/mL and 0.23 ± 0.02 µg/mL in AME at a concentration of 1 mg/mL, respectively. The contents of GFs and type I collagen in AME with concentrations of 0.1, 1, 0.5, and 1.5 mg/mL is shown in Figure 2.

Quantitative analysis of conditioned medium

As mentioned earlier, AME can affect the secretion patterns of fibroblasts in cell-laden hydrogels. Therefore, we constructed different experimental groups, which mixed both the fibroblast cells (1×10^6 cell/mL) and AME (0.1, 0.5, 1 and 1.5 mg/mL) with the collagen solution, and prepared a cell-laden, AME-loaded collagen hydrogel through a simple physical crosslinking (Fig.1). The concentrations of GFs secreted from the HDFs were measured on day 7 of the cultures. As shown in Figure 2, we observed a significant difference in concentrations of all GFs and type I collagen when we compared AME 1 mg/mL and the CM3 group (contained secretion factors of fibroblasts loaded in collagen hydrogel and AME). Moreover, the CM4 group showed a significant difference compared to the cell group (FIB) in all cases and with AME (1.5 mg/mL) in type I collagen (657 ± 34.03 µg/mL). Figure 2A shows a higher concentration of HGF in the CM2 (52.5 ± 0.76 µg/mL) and CM3 (81.2

± 0.1.02 µg/mL) groups compared to the AME 0.5 mg/mL (29.4 ± 0.60 µg/mL) and AME 1 mg/mL (58.7 ± 1.12 µg/mL) groups, respectively. The concentration of VEGF secreted from the hydrogel loaded with AME (1 mg/mL), (CM3), indicated a higher concentration (0.35 ± 0.02 µg/mL) compared to only fibroblasts (0.02 ± 0.004 µg/mL) and AME 1 mg/mL (0.23 ± 0.02 µg/mL) groups (Fig.2D). In addition, CM1 (0.14 ± 0.02 µg/mL) and CM2 (0.19 ± 0.01 µg/mL) groups showed a higher volume of VEGF compared to only 0.1 and 0.5 mg/mL of the AME (0.02 ± 0.003 µg/mL, 0.12 ± 0.04 µg/mL), respectively.

Finally, concentration of type I collagen (381 ± 18.5 µg/mL) significantly increased in CM3 group compared to AME (1 g/mL) and fibroblast-only group (342 ± 25.04 µg/mL and 0.22 ± 0.12 µg/mL).

Effects of conditioned medium on cell proliferation

To evaluate the influence of different concentrations of AME on cell proliferation, HDF cells were treated with CM containing various concentrations of AME, and the secreted substances from the 3D cultured fibroblasts were analyzed at different timepoints (24, 48, and 72 hours). Expectedly, the HDFs showed a normal spindle-shaped morphology in all groups (Fig.3A). There was a visual, upward trend in proliferation of the HDFs from the cells treated with CM1 to CM3 groups, where the concentration of AME varied from 0.1 mg/mL up to 1 mg/mL. In this context, the CM3 group revealed the highest cell count compared to the other CM groups, as well as the cell group treated with FBS as the positive control group. To evaluate the metabolic activity of the cells quantitatively, the MTT assay was also assessed at 24, 48, and 72 hours timepoints. As shown in Figure 3B, the same trend of increasing OD values was observed for CM1-CM3 samples from 24 to 72 hours. This result well confirmed the proliferative role of AME on AME-treated HDFs, where the highest effective concentration of AME was 1 mg/mL. However, we observed a lower OD value for the CM4 (0.43 ± 0.02) group compared to both the CM3 (0.79 ± 0.01) and the positive control groups (0.72 ± 0.01) (26, 32). The number of viable cells of HDFs treated with the CM3 group was counted with trypan blue under inverted light microscope and reported as 2.7×10^6, 3.51×10^6, 4.5×10^6 at 24, 48 and 72 hours, respectively (Fig.3C). These data demonstrated a significant increase in the cell number for the CM-treated HDFs compared to the CM4 and negative control groups during 48 and 72 hours timepoints, respectively. Figure 3C also showed that the number of live cells increased significantly in CM1-CM3 groups compared to the negative control at different timepoints.

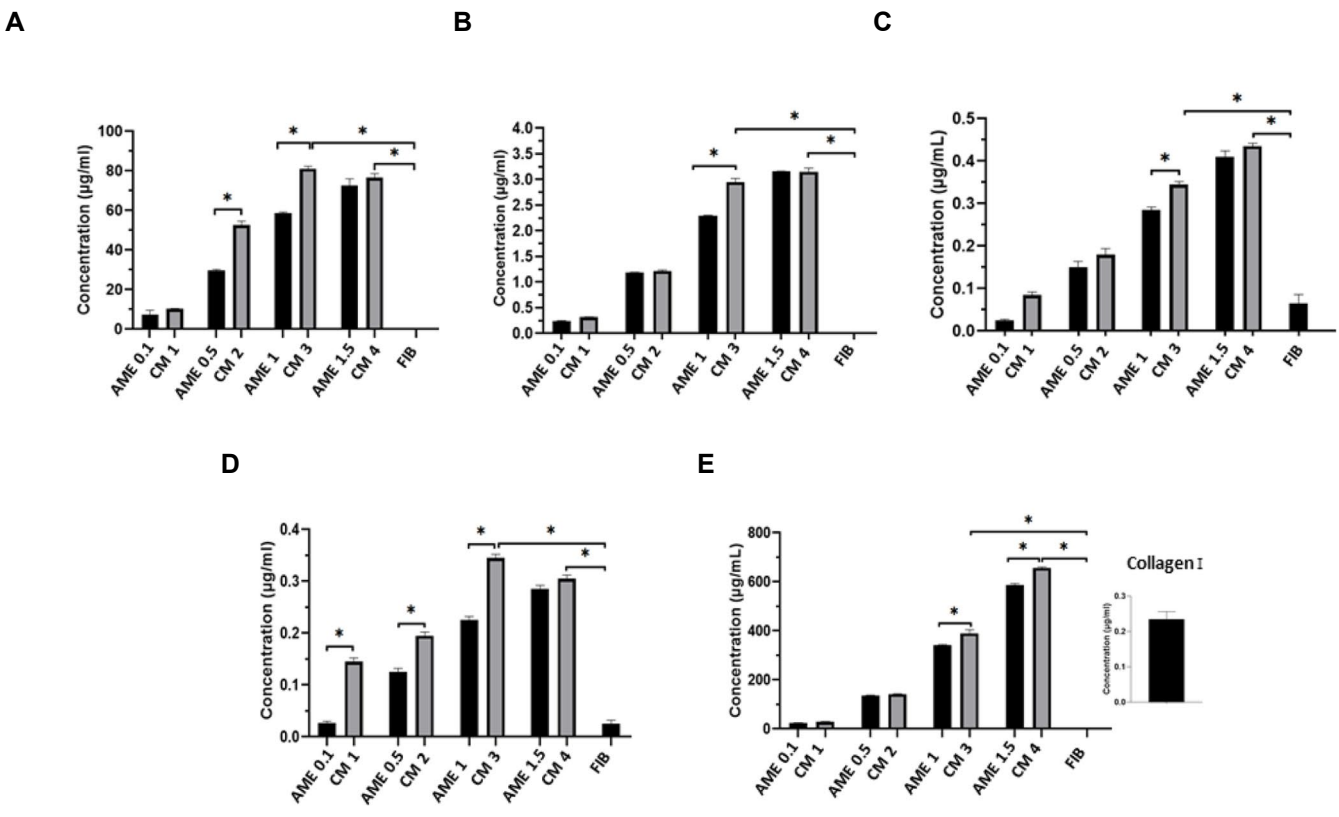

Fig.2: Quantification of concentrations of GFs and collagen type I with ELISA. The concentration of each biological factor was measured at a volume of 1 mL of AME and the related CM. **A.** HGF, **B.** EGF, **C.** KGF, **D.** VEGF, **E.** Type I collagen. Data is represented as mean ± SEM, (n=3). *; P≤ 0.05. GFs; Growth factors, AME; Amniotic membrane extract, CM; Conditioned medium, HGF; Hepatocyte growth factor, EGF; Epidermal growth factor, KGF; Keratinocyte growth factor, VEGF; Vessels endothelial growth factor, and FIB; Fibroblast.

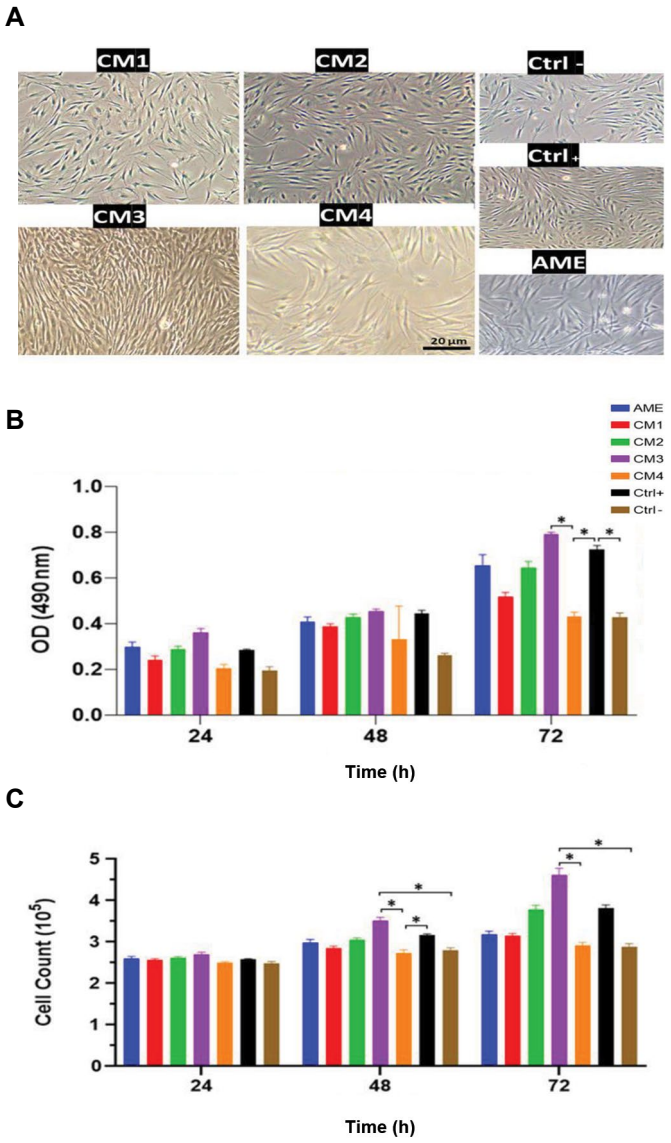

Fig.3: *In vitro* cytocompatibility of the cell-laden AME-loaded hydrogels. **A.** Microscopic images from HDFs morphology treated with different CM and control groups. **B.** Investigation of cell viability using MTT assay at 12, 48, and 72 hours timepoints. **C.** The cell number was evaluated by trypan blue at 12, 48 and 72 hours (*; P≤0.05). Data represent mean ± SEM, n=3. Ctrl-; Negative control, Ctrl+; Positive control, AME; Amniotic membrane extract, HDF; Human dermal fibroblasts, CM; Condition medium, and OD; Optical density.

Scratch assay

Cell migration is a key stage in facilitating the healing process during all phases of wound healing. Therefore, the effects of CM on *in vitro* cell migration of HDFs was studied using scratch assay, as a mimic of *in vivo* wound closure, at 0, 3, 12, and 24 hours post-injury (Fig.4A). The microscopic images from the scratched areas of the wells showed that cell migration could be observed in all groups after the 3 hours timepoint (Fig.4B). Quantitative analysis of *in vitro* wound closure illustrated that the CM3 group had the highest potential to promote cell migration compared to the other groups. According to Figure 4B, the scratched area was significantly reduced in the wells treated with CM3 group (67.1 ± 2.6%) as compared to AME (31 ± 0.8%) and CM4 groups (27.4 ± 1.3%) at 12 hours post-injury. In addition, the scratched area was significantly reduced in CM2 group (56.6 ± 0.8%) as compared to CM4 group after 12 hours. In addition, the reduction of the scratched area in CM3 group was significantly higher (96.4 ± 1.45%) than the wells treated with the negative control (62.3 ± 0.85%) and CM4 groups (71.4 ± 0.7%) after 48 hours.

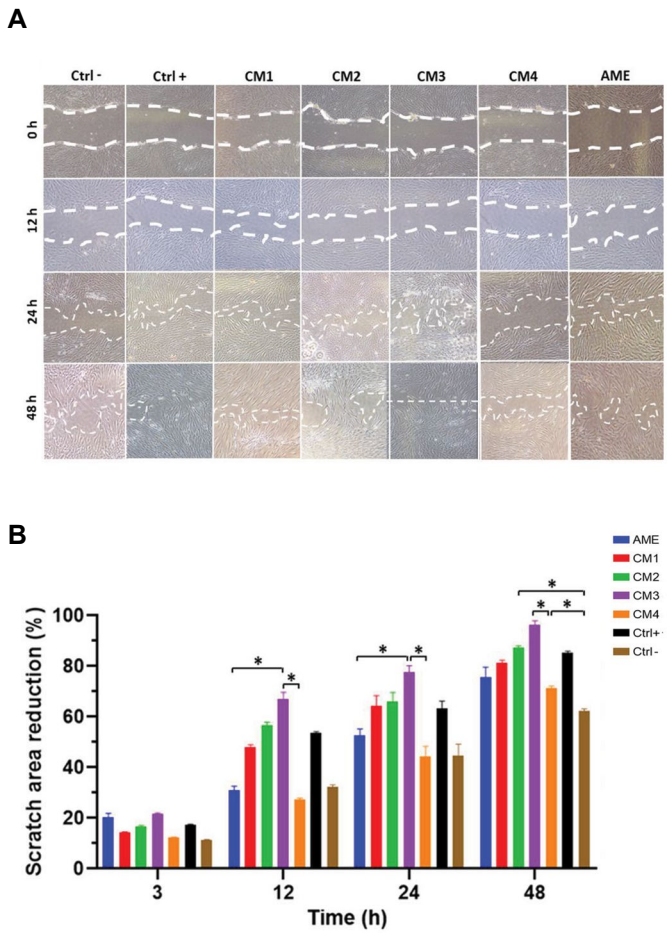

Fig.4: *In vitro* scratch assay analysis. **A.** The microscopic images illustrated different contents of migration for HDFs due to the different concentrations of AME, **B.** The amount of reduced scratched area (%) was quantified at different timepoints (*; P≤0.05). Data represent mean ± SEM, n=3. Ctrl-; Negative control, Ctrl+; Positive control, HDF; Human dermal fibroblasts, CM; Condition medium, and AME; Amniotic membrane extract,

Discussion

Combination of the cells and their signaling elements, such as GFs, on an engineered platform creates biological constructs that actively regenerate tissue (15). Previous research has shown that allogenic fibroblasts loaded into hydrogel scaffolds may provide an out-of-shelf skin substitute. Meanwhile, to have an effective cell-therapy product with this

strategy is less promising, because cryopreservation of the cells reduces viability of the cells by almost 50% and inhibits protein production by 70-98% (11, 28). Furthermore, both the time-consuming process for production of the cell-based skin substitutes and their long-term storage are both challenging (33). In state of the art, using secretory factors of cells for in situ growth and proliferation of the cells in the wound bed, to promote healing of the injured tissue may be an effective alternative method. Therefore, researchers have shifted to load the CM obtained from the fibroblasts, human umbilical cord perivascular cells (34) and adipose tissue-derived mesenchymal stem cell (35) into 3D scaffolds and probe their healing efficacy to develop new skin substitutes. In this line, it has been shown that fibroblast cells are capable of producing more GFs under the effect of exogenous GFs, which control proliferation and migration of the cells in a wound microenvironment (36).

AME contains biological components and cytokine cocktail that are effective in the wound healing process (37). We recently indicated that the presence of AME in the culture medium promotes proliferation of fibroblasts and corneal epithelium (28, 29). In this study, we aimed to investigate the effects of AME on proliferation and migration of encapsulated fibroblasts into a collagen hydrogel to optimize the effective concentration of AME for use in next-generation AME-loaded cell-based products. For this aim, cell-laden, AME-loaded collagen hydrogels were prepared through simultaneous loading of AME and fibroblasts into a collagen hydrogel, and the paracrine effect of AME on protein synthesis of the HDFs was examined. Additionally, we analyzed the secretory factors released from resulting hydrogel. The results revealed that the amount of nearly all GFs secreted in CM, which promote the growth and proliferation of the fibroblasts (38), increased by increasing the AME concentration from 0.1 mg/mL to 1.5 mg/mL. Iijima et al. (36) reported that fibroblasts produce higher amounts of VEGF and HGF when they are laminated with a membrane containing EGF at a concentration of 0.1 to 0.2 $\mu g/cm^2$ compared to a membrane treated with 0.5 $\mu g/cm^2$ of EGF. In addition, other researchers have shown that the CM, as a pool of secretory factors released from AME-loaded hydrogel, can promote regeneration of a damaged tissue by inducing proliferation, migration, angiogenesis, biosynthesis and ECM remodeling (34, 35). Likewise, our results showed that the concentration of type I collagen, which is a component of the skin ECM, increased in fibroblasts treated with different concentrations of AME (0.1-1.5 mg/mL). The ECM formation promotes proper communication between fibroblasts and it facilitates formation and maintenance of hair follicles, sweat glands, and nerves and vessels (39, 40).

Similar to recent research findings, we found that the CM extracted from cell-laden AME-loaded collagen hydrogel can stimulate proliferation and migration of the HDFs. Based on the results of MTT assay, proliferation of HDFs treated with the CM3 group were obvious compared to the cells that were treated with either CM4 or the control groups. The results of both viability and scratch assays on the CM-treated HDFs well demonstrated that proliferation and migration of the HDFs were both concentration- and time-dependent (28, 34). The best results for fibroblast migration were observed for the cells treated with 1 mg/mL of AME (CM3) group. It was also observed that HDFs treated with CM1-CM3 groups had a significant increase in proliferation compared to the CM4 group after 24 and 48 hours. Also, the number of live HDFs treated with CM1-CM3 groups during 48-72 hours showed a significant increase compared to the negative control group. However, we found that the higher concentrations of AME (< 1 mg/mL) is inappropriate for the cells, consistent with our previous results (26), which may be due to the accumulation of proliferative cytokines in CM4 group and further apoptosis of the cells. Based on our results, the concentration of 1 mg/mL of AME as an exogenous promoting substance for proliferation and migration of the HDFs was selected as the optimum concentration to be used for healing of the injured skin tissue for future studies.

Conclusion

This study was performed to pursue the *in vitro* effects of different concentrations of AME on biological factors that can be released from the cell-laden hydrogels, as well as the effects of secreted materials in CM on cell proliferation and migration. The results of this study indicated that the contents of GFs (EGF, KGF, VEGF and HGF) and the structural protein (type I collagen) secreted from the cell-laden AME-loaded collagen hydrogel may be increased in the presence of AME (0.1-1 mg/mL). The *in vitro* analyses revealed that the prepared CM from the cell-laden AME-loaded hydrogels increases proliferation and migration of fibroblasts. In this regard, the CM3 group (containing the AME and HDFs in concentrations of 1 mg/mL and 10^6 cell/mL, respectively) showed the greatest effect on secretion of GFs and collagen, as well as on proliferation and migration of the fibroblasts compared to the other groups. Our results showed that the cell-laden hydrogels reinforced by AME are potentially better solutions as pharmaceutical formulation for clinical use. However, advanced research, including *in vivo* evaluations must be performed before this new strategy could be introduced to the clinic.

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Authors' Contributions

M.A.A.; Protocol designed, conducted the experiments, and wrote the first draft of the manuscript. N.Sh.A.; Sample preparation and analyzed data. A.S.; Re-analyzed the statistical data and results. M.E., H.D.; Supervised the project and edited the manuscript. All authors read and approved the final manuscript.

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