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HUMAN GINGIVAL FIBROBLASTS RESPONSE TO HYALURONIC ACID AND ALOE VERA EXTRACT (AN IN VITRO STUDY)

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ABSTRACT

Background: The use of hyaluronic acid and aloe vera extracts in gingival therapies lacks sufficient evidence to support their significant regenerative and proliferative effects in dentistry.

Aim of the study: The study aims to compare the proliferative effects of HA and AV on gingival fibroblast cells in an in vitro study.

Material and methods: Fibroblasts were divided into three groups: control, HA-treated, and AV-treated. Cells were examined 24 and 72 hours after using H&E staining and MTT assay, PCNA staining, and RT-PCR for gene expression of AKT1, In the case of AV, the dose was 0.7973 μg/ml at 72 hours. In the case of HA, the dose was 0.379 μg/ml at 72 hours.

Results: H&E results found that both HA and AV affected HGFs proliferation; most cells showed mitosis and adhesion and appeared larger. MTT results showed viability increasing with a high significant difference. The area fraction for PCNA revealed it was lowest in the control group (0.62±0.08), followed by AV and HA treatment recording (1.87±0.11 and 2.71±0.07, respectively). In the two experimental groups, HA-treated group and AV-treated group, area fraction increased in HA than in AV with a high significant difference (p value < 0.01). HA also increased the gene expression of the AKT1 gene, with a 2.4% change in relative expression compared to the control and a 1.5% change increase in AV.

Conclusion: Both HA and AV had a proliferative effect on human gingival fibroblasts; HA has a more potent effect than AV, the difference between groups was statistically significant.

KEYWORDS: Hyaluronic acid, Aloe vera, Gingival fibroblast, MTT assay, and Gene expression.

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INTRODUCTION

Due to the limited studies and research on HA and AV in dentistry, it is a worthy point of investigation to assess the biological effects of both of them on human gingival fibroblast cells.

Aloe vera (AV), known as Aloe barbadensis, is a plant in the Liliaceae family. It possesses several qualities, including immunomodulatory, antiviral, and anti-inflammatory qualities. It is used in the treatment of lichen planus, oral submucous fibrosis, recurrent aphthous stomatitis, alveolar osteitis, periodontitis, and other conditions [1].

AV has a variety of uses in dentistry and has drawn the interest of researchers. Consequently, it has been applied in tissue engineering, reduction of plaque development, decontamination of Gutta Percha, treatment of oral lesions, endodontic lesions, periodontal disease, and among other applications. AV has been used to treat and prevent a number of additional dental and oral health issues [2], like gingivitis and periodontitis, it quickly decreases the discomfort and irritation of the gingival tissue. Clinical research has demonstrated that using AV-containing mouth rinses and dentifrices significantly reduces the development of gingivitis and plaque [3].

On the other hand, hyaluronic acid (HA), a negatively charged polyanion, is a naturally occurring linear polysaccharide. HA has exceptional physicochemical qualities, including viscoelasticity, biodegradability, and biocompatibility. In addition to its use to promote healing following dental treatments, topical HA has lately been recognized as an adjuvant treatment for chronic inflammatory disease [4]. In dentistry, HA is a multipurpose biomaterial that has many applications. Many studies have been conducted on its role in periodontal therapy, implant dentistry, endodontics, facial aesthetics, oral surgery, and orthodontics. It is a material that is attractive for use in a range of dental treatments due to its biocompatibility, regenerative properties, and antibacterial traits [5], also HA can be applied topically to the gums, oral mucosa, or other soft tissues; it reduces inflammation and speeds up recovery. When treating gingival recession or improving the aesthetics of a patient's smile, HA can be injected directly into soft tissues to improve them [6].

MATERIALS AND METHODS:

Ethical statement:

The current in vitro study was approved by the Ethical Committee of the Faculty of Dentistry, Minia University. Committee approval number (727).

Cell Culture Procedure [7]:

The human gingival fibroblasts (HGFs) cell line was cultured in complete DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in an atmosphere of 5% CO2. Growth medium was removed after 24 hours of incubation, and cells were then washed in phosphate-buffered saline solution. The wash solution was subsequently removed from the culture, and cells were treated with dissociation reagent 0.25% trypsin enzyme. The cells were incubated for five minutes at room temperature. Cells were maintained according to manufacturing protocol.

Cells were counted using a hemocytometer; the final cell density was 2 x10^5 cells/Ml. using a 96-well plate, and the volume per well was 200μL per well.

Preparation of tested materials

Hyaluronic acid (HA) powder was purchased from the Nutri Vita Shop (USA). Hyaluronic acid powder is 100% pure (Sodium Hyaluronate). Aloe vera extract powder was purchased from the Holy Natural Shop (India). Aloe vera powder is 100% pure with no chemical additives. Tested materials were prepared according to the manufacturer's instructions. Materials were mixed with distilled water. Each tested material was added to DMEM to

be added to human fibroblasts for further testing. Human fibroblast cells were divided into 3 groups; group I: a negative control group that contained cells without treatment materials, group II: a positive control group in which cells were treated with AV and group III: a group in which cells were treated with HA. Cell viability and proliferation were examined for the three groups using the following tests:

Hematoxylin and eosin staining technique:

For each treatment, cells from the three groups were divided among three sterile slides after 24 and 72 hours. Rehydrated in decreasing alcohol concentrations (100%, 90%, 75%, and 50%) after air drying and methanol fixing. The slides underwent a 5-minute cleaning in distilled water, followed by a 3-minute immersion in filtered hematoxylin stain and two distilled water washes. The slides underwent a 5-second immersion in filtered eosin stain before being rinsed with distilled water. After dipping dried slides in xylene, they were mounted using Canada balsam, covered with coverslips, and let to dry.

The prepared sections were examined and photographed using a biological inverted light microscope (ZEISS, Germany) fitted with a digital camera (Canon, Japan). Images were uploaded to the computer system to be analyzed, field selection was based on the highest number of proliferative cells, and photomicrographs were evaluated based on proliferation morphological criteria.

Immunohistochemistry (PCNA staining technique) [8]:

The slide was prepared with a 1% hydrogen peroxide solution, submerged in absolute alcohol, and incubated for 10 minutes. It was then soaked in distilled water for an additional ten minutes. The slides were treated with PCNA antibody overnight to remove background staining and enable specific binding. After washing three times in PBS, they were incubated with a secondary antibody and horseradish peroxidase for up to an hour. The slide was then dried by dissolving it in progressively higher alcohol concentrations. The prepared sections were examined and photographed using a biological inverted light microscope (ZEISS, Germany) fitted with a digital camera (Canon, Japan), Faculty of Medicine, Al-Azhar University.

The immunoreactivity for PCNA was measured using Image J software, resulting in an area fraction. The area was assessed under a 200x magnification light microscope, using areas with positively immunostained tissues for examination regardless of staining intensity.

Histomorphometric analysis of PCNA immunopositivity:

The software was adjusted to 200 X magnification for all fields of interest, and the picture was modified to show the immunopositivity brown color threshold, covered by a blue binary color to obtain the area fraction. The program was adjusted to measure the blue binary color's area fraction in relation to the measurement frame's area. The mean area fraction of the PCNA marker was calculated and tabulated for each group, with the results presented as mean values and the probability factor calculated to evaluate the results' significance (measuring area of fraction was valuable to estimate the extent of proliferation across the sample and to know the proportion of cells that are proliferating).

Cell viability and proliferation test (MTT assay) [9]:

Cells were seeded onto 96-well plates and incubated for 24 hours at 37°C. The plates were then sterilely formulated with HA and AV. Each well was then treated with MTT and DMSO, and the plates were rotated for five minutes. The absorbance of the cells was measured using an ELISA reader, and the results showed a positive correlation with the quantity of metabolically active cells. The rate of cell growth decreased when absorbance values were lower than the control cells, while greater cell growth was suggested by a higher absorption rate.

RT-PCR for gene expression of AKT1 (a marker for cell proliferation):

The National Centre for Biotechnology Information (NCBI) has been used for primer selection and alignment of Homo sapiens AKT serine/threonine kinase 1 (AKT1) as a housekeeping gene and test marker for proliferation. The NCBI website was used for primer sequence alignment through BLAST analysis and by FASTA sequence.

Primer sequence

AKT1: F 5'- TGGACTACCTGCACTCGGAGAA-3',

AKT1: R 5'- GTGCCGCAAAAGGTCTTCATGG-3'.

GAPDH: F 5'- GTCTCCTCTGACTTCAACAGCG-3'

GAPDH: R 5'- ACCACCCTGTTGCTGTAGCCAA-3'

Procedure:

The study involved extracting total RNA from HGF cells treated with HA and AV and a negative control after 72 hours using a MIRNeasy Serum/ Plasma Advanced Kit. The RNA was then purified and used to synthesize cDNA. The ReverAid RT Kit reverse-transcribed the RNA, and real-time PCR was performed on the Rotorgene RT-PCR system to measure mRNA expression. The relative gene expression was calculated using the Double Delta Ct" Method for Relative Quantification. The results provide valuable insights into the mRNA expression of HGF cells.

Statistical methods

The IBM SPSS statistics (statistical package for social sciences) software version 22.0, IBM CORP., Chicago, USA, was used to organize and statistically analyze the acquired data regarding the area of fraction for PCNA immunoreactivity, also viability percent for MTT assay, and RT-PCR for AKT-1 expression. The data were then described as means±SE (standard error). The comparing between groups was done by using the one-way ANOVA test. A P-value of ≤ 0.05 was considered significant, and anything less than 0.01 is considered highly significant. The EC50% test was done by agonist equation using GraphPad Prism program version 6.0.

RESULTS

Hematoxylin and eosin staining (H and E, original magnification 100X, oil).

In Group I (control group), fibroblasts appeared regular with normal cellular appearance. Cells exhibited an intact cell membrane. **(Fig: 1).**

In Group II (Aloe vera group), after 24 and 72 hours of treatment with AV, hematoxylin and eosin-stained sections of cultured HGFs showed large adhered cells, some of the HGFs showed proliferation and increased in number with an intact cell membrane **(Figs: 2A&2B).**

In Group III (Hyaluronic acid group), after 24 and 72 hours of treatment with HA, hematoxylin and eosin-stained sections of cultured HGFs showed large cells, most cells showed proliferation and increased in number and appeared adherent with an intact cell membrane also cells were hyperchromatic with chromatin condensation **(Figs: 3A&3B)**.

Immunohistochemical results (PCNA, original magnification 100X, oil).

Group I (control group) showed weak cytoplasmic immunoreaction for PCNA **(Fig: 4)** while Group II (Aloe vera group) showed a moderately positive cytoplasmic immunoreaction for PCNA immunostain (**Fig: 5A&B),** and Group III (Hyaluronic acid Group) showed a more positive cytoplasmic immunoreaction for PCNA immunostain **(Fig: 6A&B).**

Measuring the area fraction for PCNA revealed that the area fraction was lowest in the control group (0.62±0.08), followed by AV and HA treatment recording (1.87±0.11 and 2.71±0.07, respectively). In the two experimental groups, area fraction increased in HA treatment than in AV treatment with a high significant difference (p value<0.01) (**Table 1).**

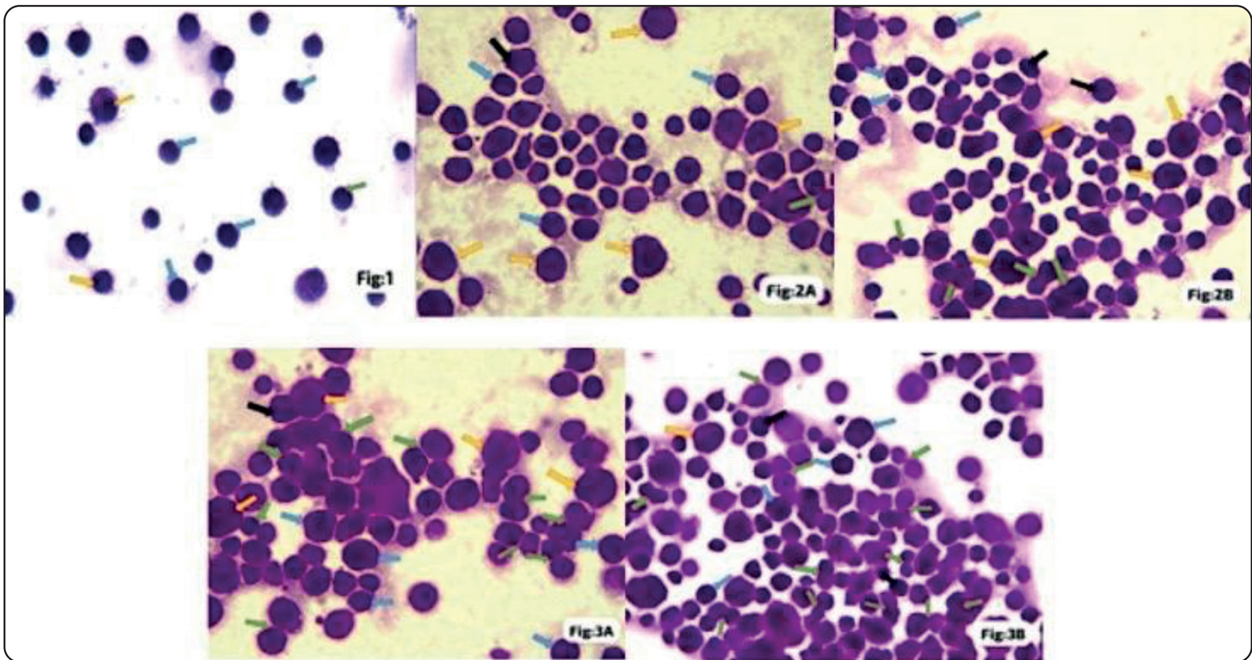
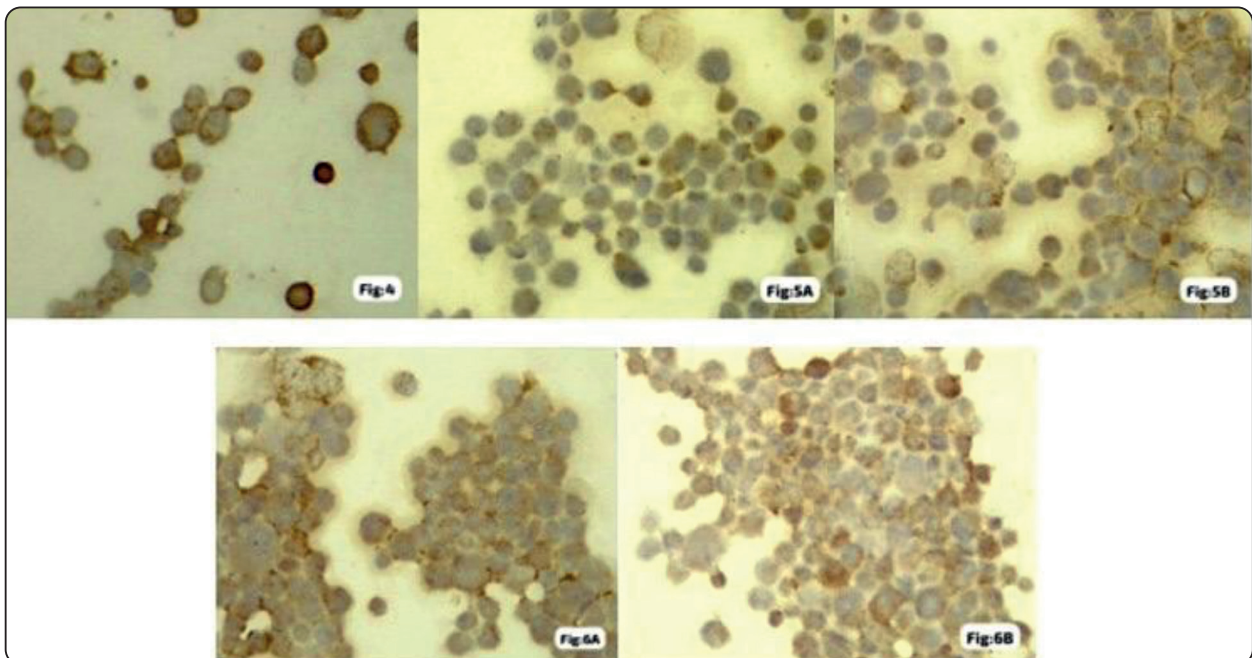


Fig: (1-3) A photomicrograph of HGFs of group I (control group) showing: normal cell morphology (green arrow), normal nuclear appearance (yellow arrow), and intact cell membrane (blue arrow). **(Figs: 2A&2B):** AV group after 24 hours and 72 hours showing increase numbers of cells, adhesion, and division of the cells (green arrow), larger cells (yellow arrows), condensed chromatin (black arrow), an intact cell membrane (blue arrow). **(Figs: 3A&3B):** HA group after 24 hours and 72 hours showing increased number of cells, division, adhesion and condensation of the cells (green arrow), larger cells (yellow arrow), condensed chromatin (black arrow), and intact cell membrane (blue arrow) **(H and E, original magnification 100X, oil)**.



(Fig: 4-6) Control group showing: weak cytoplasmic immunoreaction in HGFs. **(Fig: 5A&B):** AV group showing: brown stained positively cytoplasmic immunoreaction for PCNA. **(Fig: 6A&B):** HA group showing: much brown stained positively cytoplasmic immunoreaction for PCNA **(PCNA cytoplasmic reaction, original magnification 100X, oil)**.

TABLE (1) Comparison of mean values regarding the mean area fraction of PCNA immunoreactivity in the control group, AV group, HA group.

Area of Fraction	Mean±SE	95% Confidence Interval for Mean		Minimum	Maximum
		Lower Bound	Upper Bound		
Control	0.62**±0.08**	.41	.82	.40	.90
HA	2.71**±0.07**[a]	2.53	2.89	2.40	2.90
Aloe vera	1.87**±0.11**[ab]	1.60	2.14	1.50	2.20

a: Represent the highly significant difference versus control (P value<0.01)

b: Represent a significant difference versus HA (P value<0.05) (p value =0.017).

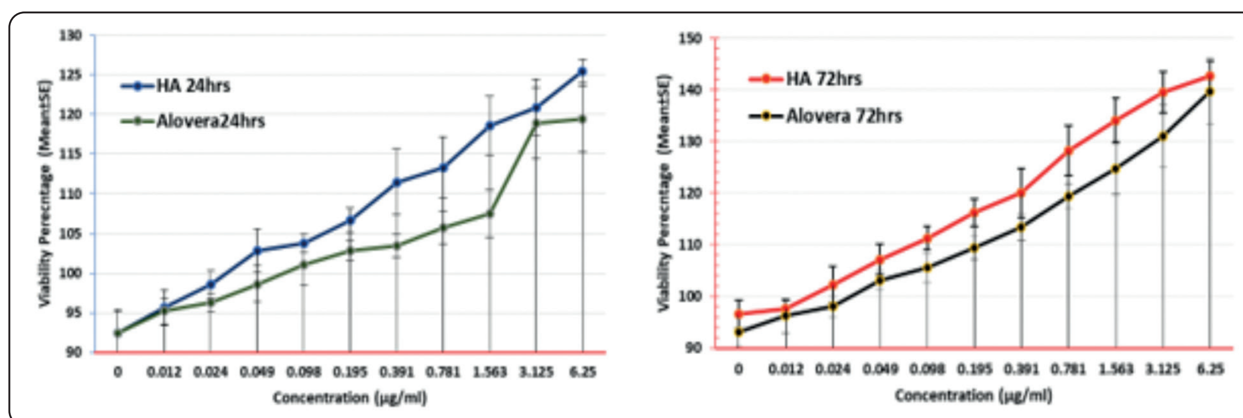


Fig. (7) Line graph comparing viability percent among Hyaluronic acid and Aloe vera at 24 and 72 hours with different concentrations.

Cell viability and proliferation test (MTT assay) results:

Cell viability was evaluated using the MTT assay after 24 and 72 hours based on the cell's viability as the follows:

The cell proliferation and viability increased according to the change in concentration in addition to the time-dependent way. Comparing HA and AV at 24 and 72 hours revealed that viability percent was higher in HA as compared to AV, and the difference was statistically significant, the viability tested in both groups significantly increased by time (time-dependent) **(Fig: 7).**

In the case of AV, the EC50% dose was 1.858µg/ml and 0.7973µg/ml at 24 hours and 72 hours, respectively. In the case of HA, the EC50% was 0.4238µg/ml and 0.379µg/ml at 24 hours and 72 hours respectively **(Fig: 8)**.

RT-PCR for expression of AKT1:

The hyaluronic acid group exhibited more expression of AKT1 than that obtained by the Aloe vera group at 72 hours, and the lowest expression was obtained by the control group. (**Table 2**), the percent of change is recorded 2.4% and 1.5% in comparison to the control group using HA and Aloe vera treatment, respectively. In the case of AV, the dose was 0.7973 µg/ml at 72 hours. In the case of HA, the dose was 0.379 µg/ml at 72 hours.

TABLE (2) Real-time PCR for AKT1 among the control group, Hyaluronic acid and Aloe vera.

Groups	GAPDH - CT Values	AKT1 - CT Values	δ CT AKT1	δδ CT	AKT1 relative expression 2^-δδ CT	% of the change in Relative Gene Expression
Control	21.8	29.5	7.8	0.0	1.0	0
HA	21.8	27.8	6.0	-1.8	3.4	2.4
Aloe vera	21.8	28.2	6.4	-1.3	2.5	1.5

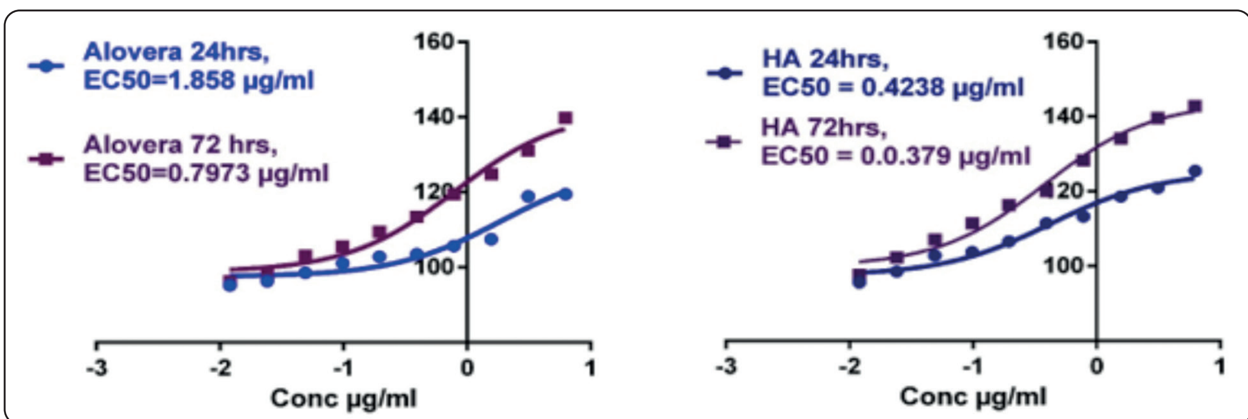


Fig. (8) Line graph showing the dose at which Viability compared in Aloe vera and HA form at 24 and 72 hours.

DISCUSSION

Aloe Vera is regarded as the “miracle plant,” the oldest known medical herb, and the most widely used one globally [10]. In vitro and in vivo studies have demonstrated the anti-inflammatory, anti-arthritic, and antibacterial qualities of the 75 active compounds that make up AV, which include vitamins, enzymes, minerals, sugars, salicylic acids, and amino acids [3]. On the other hand, HA is synthesized by nearly all cell types, meaning that it has numerous fundamental biological activities in normal biological conditions, numerous biological processes (lubrication, hydration, matrix structure, and steric interactions) as well as cellular interactions (differentiation, proliferation, development, and recognition) might include it, additionally, during the healing phase, it may have a role in tissue remodeling and regeneration, it is also intriguing for use in biomedical applications due to the compound’s anti-inflammatory, immunomodulatory, anti-diabetic, anti-aging, wound healing and tissue regeneration, skin repair, and cosmetic qualities [4]. Therefore, the current study was conducted to in vitro assess the proliferative potential of AV and HA on human gingival fibroblasts as a positive control and human gingival fibroblasts without treatment as a negative control.

The development of in vitro models is essential to improving our knowledge of cell physiology and biology, disease modeling, cancer research, medication development, toxicity evaluation, stem cell studies, regenerative medicine, and biotechnology are just a few of the fields in which these models are extremely important [11].

In vitro testing is suitable for mimicking biological reactions to materials, as compared to expensive and time-consuming animal studies, the quantity, growth rate, and other biological activities of the cells exposed to the substance are counted in order to evaluate the material's impact. They are rapid, somewhat simple, repeatable, regulated by experiment, and ethically acceptable [12].

This study employed human fibroblast cell culture because it offers a useful, repeatable, and affordable method, human fibroblasts were selected because of their relevance to therapeutic settings, they also have the benefits of being simple to isolate and growing rapidly in standard culture media. Four different techniques were used to accurately evaluate cell viability, histologic analysis using hematoxylin and eosin stain and light microscopy were used to identify unique characteristics of proliferation, and the MTT test, one of the most used screening techniques for assessing cell viability and proliferation, has also been employed since it is efficiently calibrated, accurate, and dependable [13], additionally, PCNA expression was evaluated using immunohistochemistry, since PCNA is a well-conserved protein that has evolved over time and is necessary for DNA synthesis during replication, it has been demonstrated that PCNA expression levels are linked to proliferation [14], lastly, AKT1 expression was measured using the RT-PCR method. One of the most adaptable kinase families, AKT plays a crucial role in controlling cell migration, metabolism, proliferation, and survival [15].

Our results after 24 hours and 72 hours of treatment with AV, hematoxylin, and eosin-stained sections of cultured HGFs showed large adhered cells, some of HGFs showed proliferation and increased in number with intact cell membrane. In agreement with our study, the results of an in vitro study using a human fibroblast cell line and histological examination by H&E staining showed that AV had a high proliferative effect on fibroblasts, which can be attributed to a water-soluble polysaccharide called glucomannan compound, the study also showed that the number of migrated fibroblasts in AV-treated groups was significantly ($P<0.0005$) higher than controls, and treated fibroblasts appeared more widespread than fibroblasts in the control group [16]. In a different histology study, a mouse fibroblast cell line was used, which has long been utilized as a model for eukaryotic cells that resemble human fibroblasts, the study found that AV enhanced fibroblast proliferation, which suggests that these properties of AV might promote wound healing [17].

Furthermore, an in vitro study employing human type II diabetic and nondiabetic skin fibroblast cell lines showed that AV contained a compound that neutralizes, binds with the fibroblast growth factor (FGF-2) receptor, and stimulates gap junctional intercellular communication (GJIC), which is responsible for the ability of AV to promote the proliferation of human skin fibroblasts in diabetes mellitus [18]. Additional laboratory experiments on white rats receiving AV therapy were conducted to demonstrate the impact of AV extracts on the recovery of wounds with clean skin, according to the results, the group that had AV administration possessed greater numbers of fibroblasts than the other groups, and this difference was statistically significant ($p = 0.011$) [19].

On the other hand, our results After 24 and 72 hours of treatment with HA, hematoxylin, and eosin-stained sections of cultured HGFs showed large cells, most cells showed proliferation and increased in number and appeared adherent with intact cell membrane, also cells were hyperchromatic with chromatin condensation. Consistent with our findings, a significant increase in HGF cell proliferation was seen from the first to the fifth day at HA doses of 2 mg/mL and 6 mg/mL, thus it may be proven that HA stimulates fibroblast proliferation, which is necessary for tissue regeneration and repair [20]. Furthermore, an in

vitro study revealed that HA significantly increased HDF (Human Dermal Fibroblast) proliferation, in a rabbit model of chronic rotator cuff injuries, this controlled laboratory investigation demonstrated that the combination of HA and HDFs might promote healing [21]. Additional investigation assessed the effects of two popular dermal filler materials on human dermal fibroblasts both in vitro and in vivo: hyaluronic acid (HA) and poly-L-lactic acid (PLLA). At 72 hours, HA dramatically boosted cell proliferation in comparison to the control group ($p < 0.05$) [22]. Likewise, fibroblast cell adhesion and proliferation were successfully shown by Gelatin/Hyaluronic Acid (Gel/HA) scaffolds [23]. Also, Fibroblast proliferation is modulated by HA, and the consequences of this modulation vary according to matrix interactions and cell density [24]. Additional In vitro research examines the potential of chemically modified hyaluronic acid (HA)-based hydrogels, particularly methacrylated HA (MEHA) and maleated HA (MAHA). Both MEHA and MAHA promoted the growth of important cell types involved in wound healing, such as human dermal fibroblasts [25].

Our results of the MTT assay showed that viability percent was higher in HA as compared to AV at 24 hours and 72 hours, and the difference is statistically significant. Cell viability increased according to the change in concentration in addition to the time-dependent way. The results go in accordance with [20] who observed Cell Viability After 24 hours of treatment, cell viability in HGFs treated to HA at concentrations of 1, 2, 4, 6, 8, 10, and 16 mg/mL exceeded 70%, suggesting that HA is not cytotoxic at these levels.

Additionally, A fibroblast cell line and an MTT test were used to assess the behavior of fibroblast cells, the results indicated that gelatin/HA scaffolds may be a potential material for wound healing and skin regeneration because of their excellent biocompatibility [23].

Another study assessed the effects of incorporating polysaccharides, specifically hyaluronic acid (HA) and chitosan (CH), into a polyurethane (PU) polymer to produce scaffolds that encourage fibroblast proliferation. The results indicated that the PU/HA and PU/CH/HA scaffolds were biocompatible and produced an ideal environment for fibroblast growth and proliferation. Particularly in the first phases of culture (24–48 hours), these scaffolds promoted fibroblast motility, penetration, and cell viability [26]. Furthermore, an MTT assay assessed the cytotoxic effect and cell viability of a honey and aloe vera combination on the NIH3T3 fibroblast cell line, it was found that the combination increased fibroblast cell viability, with 99.80% cell viability achieved by the combination of 0.1% honey and 0.5 μg/mL aloe vera [27].

Consistent with our MTT assay findings, immunohistochemical analysis for PCNA revealed that the area of fraction was lowest in the control group (0.62±0.08), followed by AV and HA treatment recording (1.87±0.11 and 2.71±0.07, respectively), area fraction increased in HA treatment than in AV treatment with a high significant difference (p value<0.01). These results also go in harmony with RT-PCR for AKT1 that revealed AKT1 gene expression in the HA group (2.4%) more than that obtained by the AV group (1.5%). In line with our findings, HA accelerated tendon healing in the rat model and had no cytotoxic effects on tendon fibroblasts in vitro [28].

Furthermore, polypeptide-modified HA demonstrated excellent biocompatibility in both in vitro and in vivo experiments, fibroblast cell viability and proliferation on the resurfaced material were verified by histological and immunohistochemical staining techniques, suggesting that the polypeptide-modified HA promoted cell growth and attachment [29]. Additional study showed that AV increased cell proliferation both systemically and orally, as seen by PCNA staining, this supported the theory that AV

could promote tissue regeneration by promoting the growth of epithelial and fibroblast cells [30].

CONCLUSION

This study concludes by highlighting the potential of hyaluronic acid (HA) and aloe vera (AV) in promoting fibroblast proliferation, which is essential for wound healing and tissue regeneration. The histologic analyses, MTT tests, and PCNA expression assessments demonstrated that both AV and HA had significant impacts on the proliferation of human gingival fibroblast (HGF) cells, both compounds proved to be efficient and biocompatible in promoting cell growth, however HA showed a somewhat better rate of cell survival and proliferation than AV. The findings align with previous studies, indicating that AV and HA could be promising candidates for The results are consistent with previous research, indicating that AV and HA may be potential candidates for therapeutic applications, particularly for the restoration of gingival and dermal tissue. Applications in regenerative medicine, especially for gingival tissue repair.

RECOMMENDATION

Based on the findings of this study, I recommend combining Aloe Vera (AV) and Hyaluronic Acid (HA) for enhanced results in gingival tissue regeneration and fibroblast proliferation. Both AV and HA have demonstrated significant individual benefits in promoting cell viability, proliferation, and tissue repair. However, their complementary properties, such as AV's anti-inflammatory and glucomannan-induced fibroblast stimulation alongside HA's superior biocompatibility and ability to enhance cell adhesion and proliferation, suggest that a synergistic approach may amplify their effects. Future research, including animal studies and clinical trials, is necessary to further validate the potential advantages of this combination.

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