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Production of a Synthetic Material using Cuttlebone and Investigation of its Effect on the Viability and Proliferation of Gingival Fibroblast Cells

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Objectives Treating and accelerating the healing of oral ulcers caused by various surgeries or injuries have long been important to avoid financial loss and mental harm. Fibroblasts play an important role in wound healing. The current study aimed to produce a suitable synthetic material using cuttlebone and investigate its effect on gum fibroblast cells to help accelerate the healing of oral ulcers.

Methods Chitin powder was extracted from squid bone (cuttlebone) to produce a synthetic material, which was subjected to physicochemical control tests. To make the samples, human gingival fibroblasts were cultured in a suitable medium and divided into four groups, namely chitin, gel, base (without active ingredient), and control groups. The effects of the produced synthetic material on the viability and proliferation of gingival fibroblast cells were investigated using MTT assay for 24, 48, and 120 hours. Data were analyzed using Dunnett's post-hoc test, Tukey's test, and one-way analysis of variance (ANOVA).

Results About 1.7 grams of chitin powder was obtained from 5 grams of cuttlebone powder. FTIR was used to identify chitin. A gel containing 1% chitin was selected. The results of MTT assay showed that the formulated gel had no toxicity, and the proliferation in chitin and gel groups increased over time compared to the control and base groups.

Conclusion It seems that using cuttlebone chitin to make a suitable gel-based synthetic material can accelerate the healing process of mouth ulcers.

Keywords Chitin; Wound healing; Fibroblast; Cuttlefish

Introduction

Treating and accelerating the healing of mouth ulcers caused by various surgeries or injuries have long been challenging for dentists, as such ulcers cause financial loss and mental harm. Ulcers of the mouth, tongue, and inner of cheek are often caused by minor injuries, viral infections, or even various surgeries or systemic problems. They may be painful and annoying, especially if they are visible. They may be very red or swollen, bloody, and purulent. Depending on the size, severity, and location of the ulcers in the mouth, they can make it difficult to eat, drink, swallow, speak, or breathe. [1]

Because of eating, drinking, and the presence of various microorganisms and fungi, the mouth is an environment prone to contamination, and accelerating the healing of mouth ulcers can prevent many possible negative effects, such as infections and nutritional disorders. Having a material that can effectively aid the healing of mouth ulcers would help solve the problems caused by all kinds of oral and mucosal ulcers. [2]

For a biological material to be considered acceptable, it should be biocompatible, have a natural appearance, resemble the lost tissue, be bacteriostatic and bactericidal, and most importantly, cause no toxic damage to the cells near the treated tissue. In tissue engineering, chitin extracted from cuttlebone has been used to partially or completely replace tissues by releasing bioactive compounds or affecting cell growth. [3]

Fibroblasts are very important in the healing process. It is currently accepted that fibroblasts follow the inflammatory cells in the tissue injury site and help heal the wound through synthesis. Their structural proteins also facilitate wound contraction and extracellular matrix reorganization. [4] Fibroblasts play an important role in wound healing through migration to the wound site, proliferation, and subsequent synthesis of cytokines and extracellular matrix (ECM) for wound contraction and regeneration. Fibroblasts produce ECM through the production of collagen and non-collagenous ECM proteins, for example, fibronectin, glycosaminoglycans (GAGs), and proteoglycans. Fibroblast wound healing activity is adjusted by various growth factors, cytokines and chemokines, including fibroblast growth factors, interleukins (ILs), vascular endothelial growth factor (VEGF), and transforming growth factor-beta family (TGF-β). [5] Wound healing involves the phenotypic change in fibroblasts from quiescent cells to proliferating cells and subsequently to migratory cells and then to quiescent and contractile matrix-producing cells. Fibroblasts are generally considered as the target cells of cytokines and growth factors, which makes their role in the wound healing process more prominent. [6]

Cuttlebone is a squid skeleton found in the waters of the Persian Gulf and the Sea of Oman. The chitin present in cuttlebone activates inflammatory cells and increases cell migration to the injury site, thus increasing the healing process, as tissue formation during wound healing depends on the migration of inflammatory cells as well as fibroblasts and epithelial cells. [4] The anti-bacterial and anti-fungal properties of cuttlebone have been investigated and proven in several articles. Furthermore, cuttlebone increases the production of TGF-β and VEGF by macrophages. [7] VEGF is one of the strongest stimulating factors of angiogenesis which increases the mitosis of endothelial cells. [8]

The lack of an effective material to accelerate the healing of mouth ulcers leads to the primary aim of this study. Cuttlebone is a cheap, nontoxic waste material with special characteristics and is readily available in Iran. [5] Despite all advantages, research on cuttlebone in the country is limited to few studies, none of which considering the mouth. Therefore, the information gap and lack of a suitable synthetic material to aid in the healing of mouth ulcers led to the present study which investigated the effects of a synthetic material produced using cuttlebone on gingival fibroblast cells.

Methods and Materials

This research was conducted during 2021-2022 at Tehran Islamic Azad University of Medical Sciences, School of Dentistry. In this experimental study conducted at the laboratory, attempts were made to produce a suitable synthetic material for intraoral application using cuttlebone and to evaluate its effect on gingival fibroblast cells.

The skeleton of Sepia pharaonis, a species of squid from southern Iran [9], is found in substantial amounts along the Persian Gulf beach. Thus, the cuttlebone from this species was used in the current study (NO: 610-PMP/A). [10]

Preparation of synthetic material (gel)

A. Preparation of cuttlebone powder

Cuttlebone is structured from a combination of organic-inorganic components; protein and chitin compose the organic component and calcium carbonate and calcium phosphate compose the inorganic component. In the present study, 200 grams of cuttlebone was placed in a dish and covered completely with NaoH 1N (Merck, Germany). The dish was then placed on a heater at 100 °C for one hour, after which it was washed with distilled water. This process was repeated three times until the cuttlebone protein was and the NaoH were thoroughly removed. Then, HCL 1N (Merck, Germany) was added, the dish was left for 30 minutes at room temperature, and then it was washed again three times with distilled water. Accordingly, the minerals of the powder were lost. Then, the resulting powder was kept in a sterile environment until getting completely dry and the adhesion of the powder components was removed. [9]

B. FTIR

The resulting chitin powder was inspected by Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer, England), and the graph obtained was compared with the standard FT-IR graph of chitin to ensure the authenticity of the prepared powder. [11]

C. Making a synthetic material suitable and compatible with the resulting powder

For example, assume that 120 cc of gel is required. For this purpose, carbomer 940 (Titran) and HPMC (hydroxypropyl methyl cellulose) (Merck, Germany) as gelling agents were dissolved in 80 cc of water. Then methylparaben (0.18%) (Merck, Germany) and propylparaben (0.02%) (Merck, Germany) as preservatives were dissolved in 40 cc of water. At the end, glycerin (2%) (Merck, Germany) was added to 120 cc of the combination of the two mentioned solutions as a humectant to absorb moisture. Here the gel was made with one combination of material. The same process was repeated with two other proportions of gel constituents (Table 2). To adjust the pH and make the final gel, triethanolamine (Merck, Germany) was used, and the compounds were mixed using a good mixer. Next, 1%, 2 and 3% of the active ingredient (chitin) was added to the prepared bases. [12-14]

D. Organoleptic tests

The product was examined in terms of apparent properties, such as color, smell, and transparency. During the shelf life of the product, its color, smell, and transparency should not change. [15]

E. pH measurement

The pH of the gels should be within the normal range of oral products, i.e., between 6 and 7. [16] To determine the pH, each gel was diluted at a ratio of 1:9 w/v, and the pH was measured using a pH meter (Az, Korea).

F. Mechanical stability test using centrifugation

To check the bi-phasing of the gel, an amount of each was poured into a Falcon tube and placed in a centrifuge (Kokusan, Japan) for 5-30 minutes at 2000 rpm and 25 °C. This test was repeated for three times. [17]

G. Checking for lack of growth of bacteria and fungi in the gel

The gel was placed on plates containing growth media for bacteria (Nutrinit agar/Merck, Germany) and fungi (sub-dextrose agar/Merck, Germany), put in an incubator (Fan Azma Gostar, Iran), and then evaluated after 24, 48, and 72 hours for growth of bacteria and fungi.

H. Test to determine the amount of chitin in the final gel

The amount of chitin was determined by dissolving 2 mg of the powder (chitin) in 1 ml of phosphoric acid (Merck, Germany) in a flask for 30 minutes at 50-60 °C, and distilled water was added until the volume reached 25 ml. [18]

Chitin in this solution had an absorption at 281.4 nm. To draw the standard curve, three stock solutions were prepared on three different days. From each stock solution, five series at concentrations of 57.14, 62.5, 68.96, 74.07, and 80 µg/ml were prepared, and their absorbance was read at 281.4 nm. After preparing the standard curve for three different days, 100 ± 5 mg of gel was dissolved in 1 ml of phosphoric acid, and distilled water was added until the volume reached 25 ml. Thereafter, the absorbance was read and the gel concentration determined.

I. Rheological behavior and viscosity

To check the rheological behavior of the final gel, a Brookfield viscometer (USA) with SC4-29 spindle was used and the viscosity was measured. By increasing the rotational speed from 10 to 110 rpm and then reducing it in the opposite direction, shear rate and shear stress were reported. The rheogram of the product was drawn to evaluate the rheological behavior of the gel and determine the viscosity.

J. Selection of the composition of the final synthetic material

Finally, the composition that had the appropriate quality and successful physicochemical control tests was selected as the final gel composition and evaluated for effectiveness.

Effectiveness of the final gel

A. Preparing and dividing gingival fibroblast cells into control and case groups

To prepare samples, human gingival fibroblast (HGF) was purchased from the Pasteur Institute (Iran) and cultured in Dulbeccos modified Eagle Medium (Idehzist, Iran) containing 10% fetal bovine serum (FBS/Capricorn, Germany) and 1% penicillin/streptomycin (Biosera, France). The cells were kept in an incubator at 37 °C and 5% CO_2 for 24 hours, after which, the cultured cells were placed in the secondary medium containing DMEM, the antibiotics named above, and 10% fetal bovine serum. In the following days, the samples were checked for contamination and cell growth, and if necessary, the medium was changed. As soon as the fibroblasts spread on the surface of the flask, cultured, and filled, the cells were separated from the culture surface by trypsinization. After several passages and good growth of the cells, the samples were trypsinized again and, when ready, divided into four groups: a control group and three experimental groups which were placed in the test houses, the base synthetic material, the synthetic material itself, and the prepared chitin. Then, three wells were allocated to each group. [19]

The base synthetic material included the prepared synthetic material without the active ingredient to ensure that the resulting effect was not affected by the base material.

B. Toxicity of the synthetic material and the proliferation of gingival fibroblast cells

The toxicity of the synthetic material and the proliferation of gingival fibroblast cells were investigated in the case groups, and the results were compared with the control group using MTT assay ((3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide).

After the samples were incubated for 24 and 48 hours, the cells were washed and the toxicity of the synthetic material was investigated. Five days later, cell proliferation was examined. [19]

Finally, the obtained numbers were reported on a chart made using Excel, and cell survival was expressed as a percentage. According to the percentage of cell survival, the percentage of toxicity of the synthetic material and the rate of cell proliferation were examined by SPSS 25. To statistically analyze the data, all groups were controlled on the same day by Dunnett's post-hoc test; and two-way ANOVA was used to check and compare the effects of the two-by-two compounds. Since the difference was significant, one-way ANOVA and Tukey*'s* test were used. Finally, after five days, one-way ANOVA was used to investigate the proliferation of gingival fibroblast cells by comparing the dilution with the dilution of each group, and Tukey's test was used to compare the groups.

Results

Cuttlebone

A. Preparation of powder

The required chitin powder was extracted after the cuttlebone powder was prepared (NO: 610-PMP/A.cuttlebone). About 1.7 g of chitin powder was obtained from 5 g of cuttlebone powder. The extracted powder was almost white and odorless. FTIR was used to identify the resulting powder (chitin).

B. FTIR

The resulting powder was converted into KBr discs using potassium bromide and IR press, and FTIR with an infrared spectrophotometer of 400-4000 cm^{-1} was used.

As shown in Figures 1 and 2, the spectrum obtained from the examination of the chitin powder had five main peaks, which are the same as the standard chitin spectrum (Table 1).

Preparation of gel

A. Preparation of the base

Carbomer 940 and HPMC (1% in total) were prepared at three different ratios, according to the mentioned stages (Table 2).

B. Preparation of gel containing the active ingredient

The active ingredient (chitin) amounts of 1%, 2%, and 3% was added to the base of the prepared gel.

C. Selection of the composition of the final synthetic material

Chitin has some positive partial charge. [20] For this reason, two gels at equal and double ratios of carbomer (negative charge) were not stable compared to HPMC; however, the base was stable at the 1:2 ratio of carbomer:HPMC. Moreover, the base containing 3% of the active ingredient was unstable because of its exit from the gel state. Therefore, the bases containing 1% and 2% of the active ingredient in Formula 2 (Table 2) were stable, and stability tests were performed.

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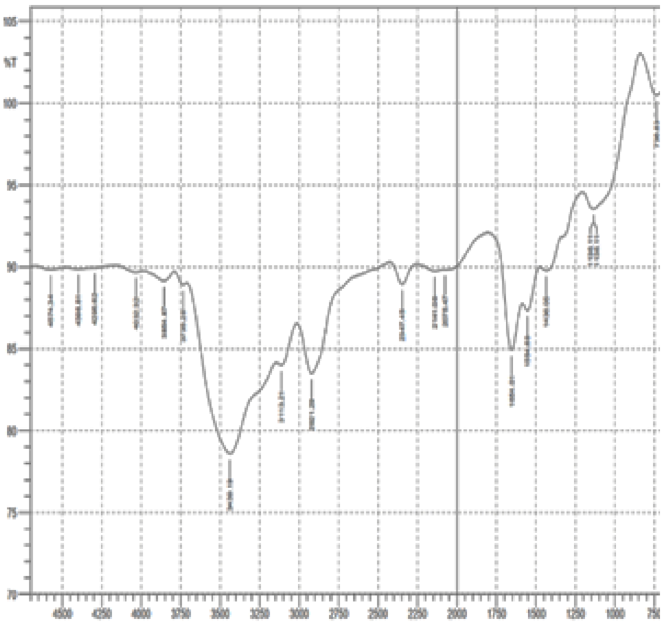


Figure 1: FTIR of standard chitin

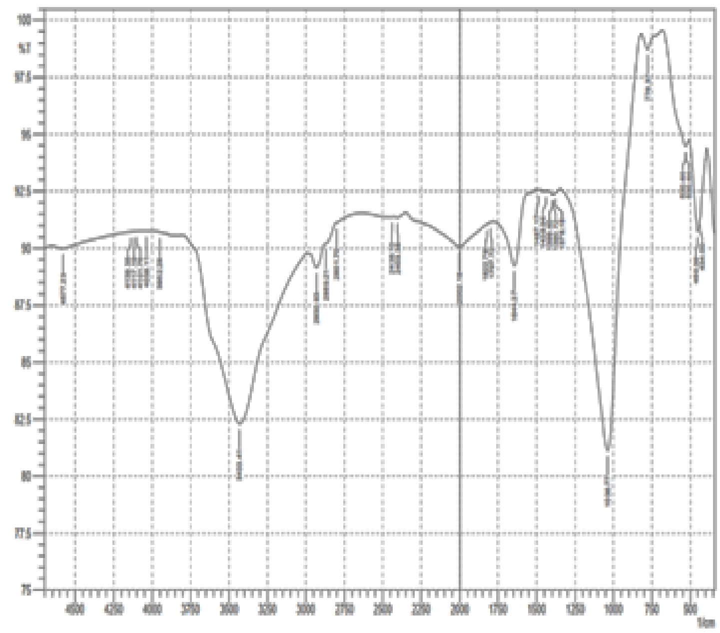


Figure 2: FTIR of chitin powder obtained from cuttlebone

Table 1- Comparison of FTIR of standard chitin powder and chitin powder obtained from cuttlebone

Peak	A large number of C-O bonds	NH bending vibration	carbonyl amide group	NH vibrational stretch	OH vibration stretching
standard chitin	1136 cm^{-1}	1554 cm^{-1}	1654 cm^{-1}	2921 cm^{-1}	3439 cm^{-1}
chitin powder obtained from cuttlebone	1036 cm^{-1}	1487 cm^{-1}	1644 cm^{-1}	2869 cm^{-1}	3433 cm^{-1}

Table 2- Percentage of formulation components

Formula	(%) Methylparaben	Glycerin (%)	(%) Propylparaben	*Carbomer:HPMC ratio	Water (%)
1	0/18	2	0/02	1:1	60
2	0/18	2	0/02	2:1	60
3	0/18	2	0/02	1:2	60

*The sum of carbomer and HPMC is 1%.

D. Quality control tests

I. Organoleptic tests

The prepared gel was colorless and odorless and had a suitable transparency.

II. pH measurement

Using a pH meter, the pH of the gel containing 1% and 2% of the active ingredient (chitin) was 6.2 and 6.6, respectively which was within the allowable limit.

III. Mechanical stability test

The gel containing 2% chitin became biphasic after 12 minutes in the centrifuge.

IV. Selection of the preferred compound

The gel containing 3% chitin was not stable because of the reduction in viscosity. The gel containing 2% chitin was biphasic in the mechanical stability test. Therefore, the gel containing 1% chitin was selected as the preferred compound.

V. Checking for a lack of growth of bacteria and fungi in the gel

No signs of bacterial or fungal growth were observed in the plates containing bacterial and fungal culture media.

VI. Rheological behavior and viscosity

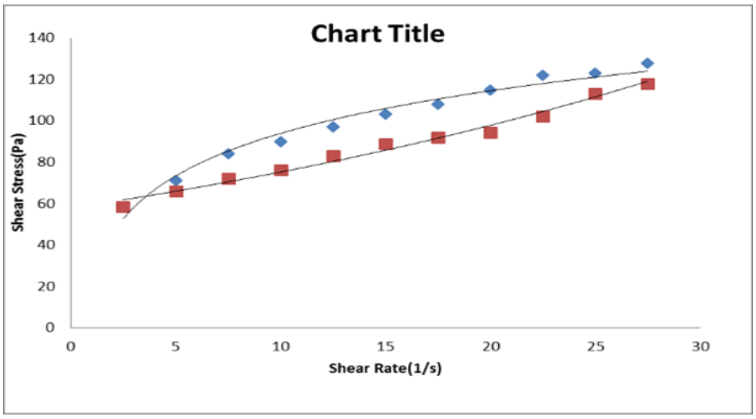
The round trip rheogram of the gel was obtained using a viscometer as follows (Tables 3 and 4). The behavior of the selected formulation of gel was identified as non-Newtonian in the subset of plastic and thixotrope (Diagram 1).

Table 3- Results of the forward rheogram of the gel containing 1% of the active ingredient (chitin)

Step point	Speed (RPM)	Torque (%)	Viscosity (Pa.s)	Shear stress (Pa)	Shear rate (1/s)
1	10	28/6	23/43	58/37	2/5
2	20	32/2	14/7	71	5/0
3	30	35/3	10/89	84	7/5
4	40	36/9	8/76	90	10/0
5	50	38/7	7/76	97/02	12/5
6	60	42/3	6/88	103	15/0
7	70	45/4	6/15	107/92	17/5
8	80	48/1	5/81	114/83	20/0
9	90	49/8	5/62	122	22/5
10	100	50/4	4/32	123	25
11	110	51/6	4/15	137/73	27/5

Table 4- Results of the backward rheogram of the gel containing 1% of the active ingredient (chitin)

Step point	Speed (RPM)	Torque (%)	Viscosity (Pa.s)	Shear stress (Pa)	Shear rate (1/s)
11	110	45/8	4/32	117/82	27/5
12	100	43/9	4/52	113	25/0
13	90	42/1	5/01	102	22/5
14	80	41/8	5/11	94/18	20/0
15	70	38/9	5/32	91/86	17/5
16	60	38/1	6/02	88/75	15/0
17	50	37/8	6/61	83	12/5
18	40	36/4	7/68	76/16	10/0
19	30	35/2	9/58	72/11	7/5
20	20	34/2	13/32	66/04	5/0
21	10	33/6	23/43	58/37	2/5



Forward ◊ - backward □

Diagram 1: General rheogram of the gel containing 1% of the effective substance (chitin)

VII. UV spectrum and standard curve and determination of the concentration

According to the standard method, the chitin standard curve was drawn on three different days (Diagram 2). Then, the concentration of the gel was determined by dissolving 100 ± 5 mg of gel in 1 ml of phosphoric acid and distilled water to a volume of 25 ml. Thereafter, the absorbance was read and the concentration determined.

After drawing the standard curve (Table 5 and Diagram 3), the absorbance of the prepared samples was read on three different days. Absorbance of the samples at 281.4 nm was reported to be 0.410, 0.423 and 0.419, indicating a concentration of 42.5 ± 0.5 micrograms/ml of chitin. This concentration indicated that the gel contained 1% of the active ingredient (chitin powder)

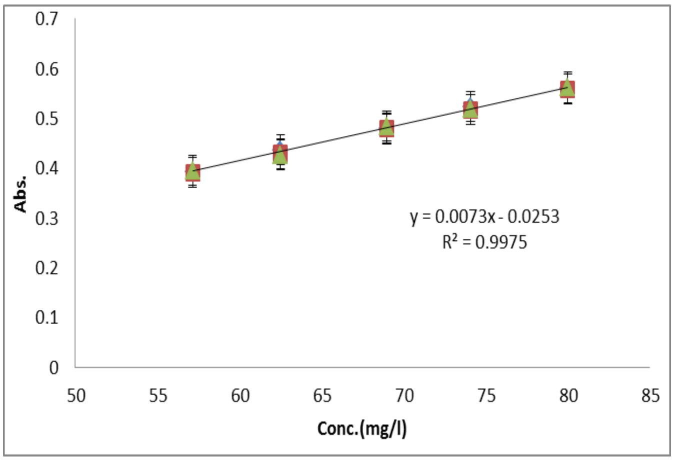


Diagram 2: UV-SPECTRUM spectrum

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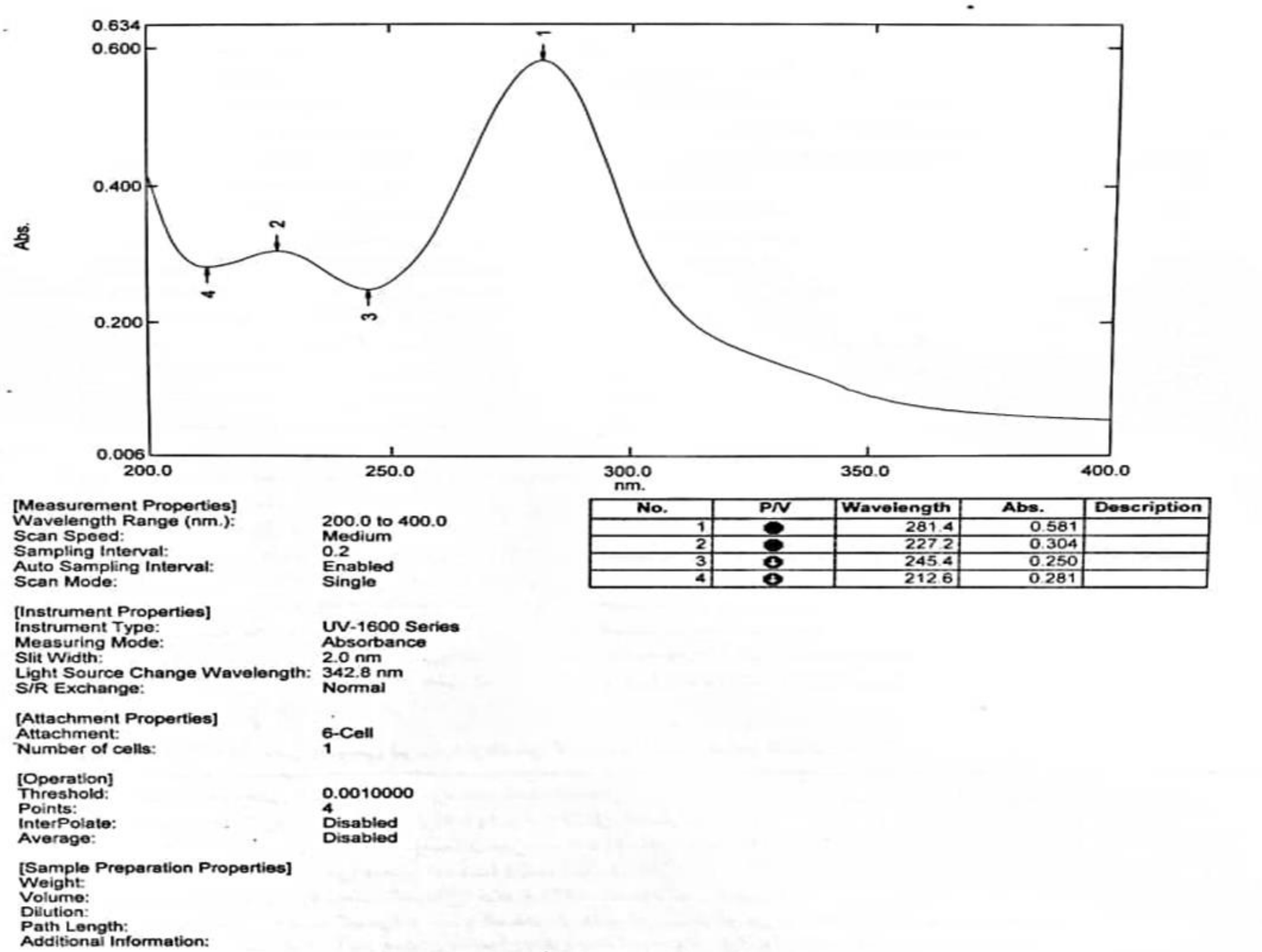

Diagram 3: Standard curve of the prepared gel

Table 5- Absorption of different concentrations in Landa Max for the prepared gel

Conc	Absorbed on the first day	Absorbed on the second day	Absorption on the third day
57/142	0/392	0/391	0/395
62/5	0/436	0/429	0/426
68/965	0/479	0/48	0/484
74/074	0/523	0/517	0/518
80	0/559	0/558	0/562

Effectiveness and toxicity of the final gel

In the current study, the effects of chitin, base, and prepared gel on the viability and proliferation of gingival fibroblast cells were investigated through the MTT assay.

According to ISO10993-5, 1 mg of chitin and 100 mg of gel and base were placed in cell wells and, for more detailed analysis, dilutions of 1.2, 1.4, and 1.8 of each group was examined.

The toxicity of the three compounds was determined by MTT assay. After 24, 48, and 120 hours, the results showed that the viability of all groups was above 70%; according to ISO10993-5, a reduction of less than 30% in cell viability compared to the control group was considered as biocompatible and safe. To statistically analyze the data, all groups were compared with the control on the same day using Dunnett's post-hoc test.

Each group was compared with the control group on the same day. After 24 hours, a significant difference was observed between the groups of dilution 1 of chitin, dilutions of 1, 1.2, and 1.4 of the base gel, and dilutions of 1, 1.2, and 1.4 of gel and the control group ($p \leq 0.05$). After 48 hours, a significant difference was observed between all groups and the control group ($p \leq 0.05$). On the fifth day (120 hours), a significant difference was observed between the groups of dilutions 1 and 1.2 of chitin, dilutions 1.2 and 1.4 of the base gel, and dilutions 1 and 1.2 of the gel and the control group, yet no significant difference was observed among the other groups. After 120 hours, cell proliferation was reduced by reducing the dilutions of chitin and the prepared gel. The highest cell proliferation after 120 hours was related to 1 mg of chitin and 100 mg of the base gel and the produced gel. After five days, cell proliferation had increased significantly in the groups containing chitin and the produced gel compared to the control group.

Next, the control group was omitted, and the effects of two compounds on cell proliferation at intervals of 24, 48, and 120 hours were investigated and compared using two-way ANOVA. Because the difference was significant, one-way ANOVA and Tukey's test were used. According to the analysis performed in the first 24 hours (Table 6), a significant difference existed between the cell proliferation results in the gel and the chitin groups ($p \leq 0.05$), despite the fact that no significant difference was seen between the gel and the base gel groups. It can be concluded that in the first 24 hours, the produced gel performed more similarly to the base gel than to chitin. After 48 hours, no significant difference was observed in cell proliferation between the chitin and the produced gel groups; unlike the first 24 hours, however, a significant difference was observed between the chitin and the base gel groups ($p \leq 0.05$). After 120 hours, the gel group showed no significant difference with the chitin group but a significant difference with the base gel group, indicating an increase in proliferation in the groups containing chitin and gel in comparison with the group containing the base gel ($p \leq 0.05$).

Table 6-. Pairwise comparison of the studied groups according to cell proliferation after 24, 48 and 120 hours

time	material		Mean Difference (I-J)	Std. Error	**Sig. (P value)**
c.v.24h	chitin	gel.base	6.2573*	1.72159	0.004*
		gel	5.3801*	1.72159	0.012*
	gel.base	gel	-0.8772	1.72159	0.867
c.v.48h	chitin	gel.base	4.2254*	1.37946	0.014*
		gel	-0.1878	1.37946	0.990
	gel.base	gel	-4.4131*	1.37946	0.010*
c.v.120h	chitin	gel.base	24.6414*	1.83823	0.000*
		gel	0.3376	1.83823	0.982
	gel.base	gel	-24.3038*	1.83823	0.000*

* The two compared groups have a significant difference.

After five days, one-way ANOVA was used to investigate the proliferation of gingival fibroblast cells by comparing the dilution with the dilution of each group, and Tukey's test was used to compare the groups. According to the results, at concentrations of 1, 1.2, and 1.4, no significant difference existed between the chitin and the gel group, but both differed from the base gel group ($p \leq 0.05$). No significant difference was observed between any of the groups at the concentration of 1.8. The analysis results also showed that by reducing the dilution of the prepared gel and chitin, their effect on cell proliferation was also reduced (Table 7).

Table 7- Comparison of cell proliferation after 120 hours for 1, 1.2, 1.4, and 1.8 dilution of materials

	material		Mean Difference (I-J)	Std. Error	**Sig. (P value)**
Dilution 1	chitin	gel.base	41.51899*	3.73856	0.000*
		gel	0.84388	3.73856	0.972
	gel.base	gel	-40.67511*	3.73856	0.000*
Dilution 1/2	chitin	gel.base	41.51899*	3.73856	0.000*
		gel	0.84388	3.73856	0.972
	gel.base	gel	-40.67511*	3.73856	0.000*
Dilution 1/4	chitin	gel.base	13.33333*	4.02004	0.037*
		gel	-3.20675	4.02004	0.718
	gel.base	gel	-16.54008*	4.02004	0.015*
Dilution 1/8	chitin	gel.base	6.91983	4.08564	0.282
		Gel	1.85654	4.08564	0.894
	gel.base	Gel	-5.06329	4.08564	0.475

* The two compared groups have a significant difference.

Discussion

Mouth ulcers are generally painful and annoying lesions that are related to various conditions such as trauma, surgery, and various systemic problems in the oral cavity. These lesions can cause a lot of mental and physical damage to patients. [21] Wound healing is a systematic biological process characterized by four overlapping stages, namely homeostasis, inflammation, proliferation, and regeneration. [22] After injury, the oral mucosa undergoes a complex sequence of biological healing processes to restore homeostasis. The lack of successful treatment for oral mucosal ulcers has influenced clinicians to explore alternative therapies and potential auto-therapies to enhance intraoral healing. [23] Fibroblasts play an important role in wound healing through migration to the wound site, proliferation, and the subsequent synthesis of cytokines and ECM for wound contraction and regeneration. [24]

In the current study, it was first attempted to produce a synthetic material suitable for increasing cell proliferation by chitin extracted from cuttlebone. Then, the effect of the synthetic material on the viability and proliferation of gingival fibroblast cells was measured through MTT assay. The toxicity of the gel formulated was investigated using MTT assay 24, 48, and 120 hours after the culture of gingival fibroblast cells, and the results showed no toxicity compared to the control group. Moreover, the survival in all groups was above 70% (according to ISO10993-5). Varoni et al. used a three-layer chitosan-based scaffold for multi-tissue periodontal regeneration and reported that more than 90% of all primary human periodontal cell populations survived (gingival fibroblasts, osteoblasts and fibroblasts of PDL) as well as active cell metabolism in collagen deposition and alkaline phosphatase assays. [25] Raquel Gallardo-Rivera et al. showed that the polyelectrolyte complex of aloe vera, chitin, and alginate causes survival (up to 90%) and migration of human fibroblasts and lymphocytes [26], which is consistent with the results of the current study.

The proliferation of fibroblast cells was evaluated using MTT assay five days after culture. The tested groups showed that proliferation in the chitin and gel groups increased over time compared to the control and base gel groups. Cell proliferation in the chitin group increased from 86.7836% in the first 24 hours to 132.3207% after five days and in the gel group from 84.6784% to 130.4641%, while the average cell proliferation in the control group (100%) was reported during all 3 periods. According to the MTT assay results, cell proliferation after five days was higher in the chitin group (115.4430) than the gel group (115.1055) which, according to statistical analysis, is not significantly different ($p>0.05$). Moreover, by further diluting the prepared gel and chitin, their effect on cell proliferation was further decreased, such that no significant difference was observed between any of the groups at the concentration of 1.8 (Table 7). According to the statistical analysis of the data, the resulting cell proliferation effect is most certainly not affected by the base of the gel material but only by the final synthetic composition.

According to previous studies, chitin extracted from cuttlebone powder has antibacterial and antifungal properties. Ramasamy et al. studied the antimicrobial potential of polysaccharide extracted from cuttlebone and methanol extracted from fish bodies. Their results showed that both had antibacterial properties against Pseudomonas aeruginosa, Staphylococcus aureus, salmonella, vibriocholera, streptococcus, candida tropicalis, candida albicans, and penicillium italicum. [27] Yazdan Panah et al. investigated the antibacterial and antifungal properties of cuttlebone and concluded that cuttlebone had antibacterial and antifungal effects on Klebsiella oxytoca and Aspergillus flavus. [28]

Lee et al. examined the effects of chitin extracted from cuttlebone on a mouse macrophage cell line. Their results showed that chitin increased the expression of MMP1. Many factors, including tumor necrosis factor, transforming growth factor beta and vascular endothelial growth factor, were observed to become increased and significantly affect wound healing. [5]

Qinyue Xie et al. evaluated the performance of 6-carboxyl chitin for wound healing and concluded that 6-carboxyl chitins can significantly stimulate the proliferation of human skin fibroblasts (HSF) and human keratinocytes (HaCaT) and have concentration-dependent biological activities. By reducing the dilution of the target substance, its effect was also reduced. These chitins can also stimulate macrophages and fibroblasts to secrete growth factors. Therefore, it can be expected that 6-carboxyl chitin is an active substance for wound healing. [29] According to the results of the current study, reducing the dilution of chitin and gel will reduce their effect on cell proliferation, and chitin can be an active substance for wound healing.

Shanmugam made collagen-chitosan film using chitosan of cuttlebone and investigated its effect on wound healing. The collagen-chitosan film was prepared using acid-soluble collagen and chitosan by the method of Montoya et al. Its effectiveness on healing a wound created on the skin of large laboratory rats was investigated. Histopathological observations showed a high migration of inflammatory cells towards the wound site in the film-treated group, leading to an increase in the proliferation of fibroblasts and collagen, and accelerating wound healing. [30] The current study also showed that the gel increases the proliferation of gingival fibroblast cells.

To confirm the increased cell proliferation of chitin and the gel, a study conducted by Jung et al. can be referred, in which the effect of nanofibers prepared from chitin extracted from cuttlebone on wound healing of the skin of laboratory rats was investigated. The resulting chitin powder was fixed on the nanofibers. Then, a wound was made using a punch, and the pathological and morphological factors were examined. The results showed that the nanofibers inhibited the inflammatory response and reduced edema and exudate secretion in the treatment groups compared to the wound group. After 14 days, there was no exudate, and the wound was healed by reducing the radius of the wound. Furthermore, the collagen deposition and proliferation of fibroblast cells was significantly higher in the group treated with nanofibers than the wound group.

In addition to its antimicrobial, antiviral and antifungal properties, chitin of cuttlebone is non-toxic and non-allergenic. It reduces inflammation and pain and heals wounds by adsorption mechanism. [20, 31] Chitin increases cell proliferation [32] and collagen synthesis [33, 34] and, in addition, increases chemotactic activity and activates complement components C_3 and C_5. [35] By reducing inflammation, chitin causes wounds to heal faster. [36]

Chun et al. investigated the effects of carboxymethyl chitin (CMCT) and chitosan based on bone induction on human periodontal ligament stem cells (hPDLSCs), i.e., cultured cells for tissue engineering therapy of alveolar bone regeneration. To clarify the effect of carboxymethyl chitosan (CMCS) and CMCT on osteogenic differentiation, hPDLSCs were isolated and treated by CMCTS or CMCT. The researchers also measured cell viability and proliferation capacity and determined the expression levels of classical molecules related to ossification, including alkaline phosphatase (ALP), phosphoprotein 1 (OPN), family transcription factor (Runx2), and osteocalcin (OCN). Mineralization levels were detected by Alizarin red staining. The results showed that both CMCTS and CMCT treatments had the maximum promotion ability for the viability of hPDLSCs below the concentration of 100 μg/mL, while CMCT significantly improved the mineralization of hPDLSCs. CMCTS induced the high expression of several factors, including ALP, Runx2, OPN, and OCN, while the little bone-inducing bioactivity of CMCT was mainly because of ALP. Therefore, CMCTS and CMCT may be promising materials for periodontal regeneration. Therefore, our formulated gel may have a good effect on the regeneration of bone and periodontal tissues; however, further studies are required. [37]

The present study aimed to provide a gel formulation containing purified cuttlebone powder and to investigate its effectiveness on the proliferation and viability of gingival fibroblast cells. For this purpose, chitin powder was prepared from cuttlebone purchased from the Bushehr city. After evaluating and reviewing various formulations, the topical gel containing 1% of the active ingredient was selected as the preferred formulation.

Physical and microbial control tests showed that the prepared products were stable to physical changes, and no effect of the growth of microorganisms was observed. The prepared gel, with its suitable apparent properties, can be used as an acceptable product for topical use. The result of the pH test showed that the pH of the product was within the allowable range. The rheological behavior of the selected gel was shown to be non-Newtonian, plastic, and thixotropic. After five days, no significant difference in cell proliferation was observed between the group tested with chitin and the formulated gel; however cell proliferation had increased significantly in the groups containing chitin and gel compared to the control group (Table 7).

Therefore, it can be concluded that the topical synthetic material of cuttlebone in the form of gel can be used to reduce the overall process of wound healing, particularly regarding mouth ulcers, by increasing cell proliferation. It minimizes infection with its antibacterial and antifungal properties, and its anti-inflammatory properties and induced cell proliferation lead to faster wound healing and reduce trauma.

Limitations

- Working with chitin is difficult, because it is a polymer and is insoluble in water.
- All chitin solvents are dangerous, so working with them requires high precision and sensitivity.
- Creating laboratory conditions similar to those in the oral environment makes the results generalizable. Therefore, it is necessary to investigate research variables under in vivo conditions.

Suggestions

- The effect of the formulated gel on the healing process of bone and periodontal cells should be investigated.
- The effects of the formulated gel should be investigated under in-vivo and clinical conditions, and the results may help the industrial production of this gel.

Conclusion

Evaluating the results of the synthetic gel on cell proliferation and viability showed that the gel can increase cell proliferation. Since it plays a role in wound healing, fibroblast proliferation, and the fibroplasia process, the gel can be assumed to accelerate wound healing. According to the current results, it seems that after using the gel for five days, significant results in wound healing occur. Therefore, it is suggested that the gel be used in laboratory clinical studies (animal and then human). Furthermore, it can be concluded that the formulated gel has no toxicity and can increase the growth and proliferation of gingival fibroblast cells, which can accelerate the wound healing process.

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Conflict of Interest

No Conflict of Interest Declared ■

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