Advances in Materials Science and Engineering

Natural and Biocompatible Materials in Dental and Craniofacial Tissue Engineering

Lead Guest Editor: Hamid Tebyaniyan
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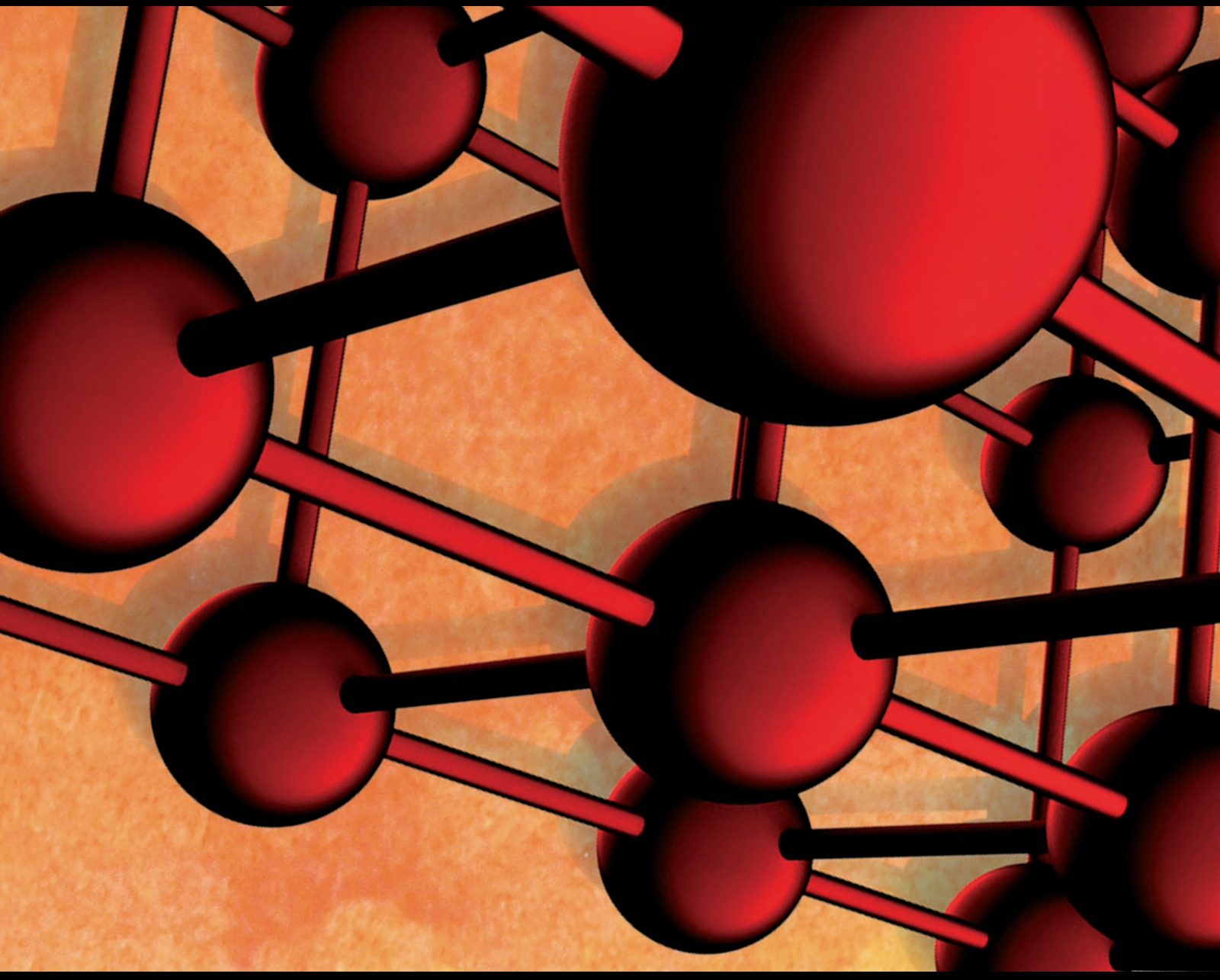

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Research Article

Biosynthesis of Selenium Nanoparticles and Evaluation of Its Antibacterial Activity against Pseudomonas aeruginosa

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Background. A fundamental component of innate immunity is represented by skin that acts as a first aid against infection. The skin's epithelial barriers, respiratory tract, and eyes directly contacting with the external environment have incremented the probability of infection. The opportunistic pathogen *Pseudomonas aeruginosa* causes various infections in immunocompromised hosts. In addition, one-third of *P. aeruginosa* clinical isolates are resistant to three or more antibiotics. Lately, lots of researchers concentrate on halophilic microorganisms due to affordable novel biomolecules. One of these biomolecules is metal nanoparticles. MNPs exhibited antimicrobial functionality against a variety of microbes. Amidst MNPs, SeNPs are one of the most extensively studied. In this study, halophilic bacteria from solar saltern were employed for the biosynthesis of SeNPs. *Aim*. This study aimed to evaluate the antibacterial properties of SeNPs which are synthesized by halophilic microorganisms. *Result*. The NPs were synthesized by *Halomonas eurihalina* intracellularly. The produced SeNPs were identified through various assays such as UV-Vis spectroscopy, XRD, DLS, FTIR, and SEM. UV-Vis spectroscopy confirmed the presence of SeNPs. In addition, the average particle size of SeNPs was 260 nm. FTIR confirmed the presence of the capping agent to inhibit the aggregation of SeNPs. Also, synthesized selenium nanoparticles have a natural crystalline nature that is verified by XRD. SEM also revealed the spherical shape. Furthermore, SeNPs represented significant antibacterial activity against *P. aeruginosa*. *Conclusion*. According to the obtained result, biosynthesized SeNPs demonstrated remarkable characteristics that make them profitable nonantibiotics and also decrease the morbidity and mortality associated with tissue infections.

1. Introduction

Pseudomonas aeruginosa as gram-negative bacteria are found in various natural habitats since they can simply adapt to various circumstances. As a result of the ability for quick adaptation, they are identified as opportunistic pathogens [1, 2]. The opportunistic pathogen *Pseudomonas aeruginosa* causes various infections in immunocompromised hosts. The most significant infections are related to soft tissues, such as burn and chronic wounds [3]. A fundamental component of innate immunity is represented by skin acting as a first aid against infection. It activates cell-mediated and humoral immune responses [4]. Evidence indicates that *P. aeruginosa* causes epithelial damage (Evidence for Epithelial Integrity Changes by *Pseudomonas aeruginosa*). Moreover, it weakens mechanisms for post-injury epithelial repair (Evidence for Epithelial Repair Impairment by *Pseudomonas aeruginosa*). In fact, uninfected epithelia can set up an organized procedure of complex mechanisms for restoring postinjury epithelial integrity and function. Meanwhile, the capability of epithelial tissues to adequately repair is restrained by the presence of *P. aeruginosa* resulting in altered organ function and persistent infections. Several studies have presented the evidence for the negative effect of *P. aeruginosa* on repair processes and epithelial integrity in the world. In these studies, different species, pathologies, epithelia, and organs were investigated. Thus, effective postinjury repair mechanisms are vital for maintaining epithelial integrity. However, these healing procedures can be inadequate for

restoring epithelial integrity, remarkably in infectious circumstances. Since *Pseudomonas aeruginosa* infections in cutaneous and corneal principally cause disabilities, hospitalizations, and mortality in the world, they have attracted particular concerns [5]. Bacteria normally included in skin infections are P. *aeruginosa*, MRSA, Acinetobacter spp., *E. coli*, and coagulase-negative staphylococci, such as *Staphylococcus lugdunensis* and *Staphylococcus epidermidis* [4]. Different organs are infected by *Pseudomonas aeruginosa* like skin, eye, ear, heart, soft tissue, blood, or joints and bone and respiratory, gastrointestinal, urinary, as well as central nervous systems. The skin's epithelial barriers, respiratory tract, and eyes directly contacting with the external environment have incremented the probability of infection. Therefore, corneal, cutaneous, alveolar, and airway infections should be concerned about their prevalence. Mostly, the cutaneous epithelium's P. *aeruginosa* infections happen after injuries. Therefore, patients with chronic cutaneous wounds and burn victims, a prevalent complication of diabetes, are mainly at higher risks of developing *P. aeruginosa* infections [1, 5]. In addition, one-third of *P. aeruginosa* clinical isolates are resistant to three or more antibiotics, including third-generation cephalosporins and imipenem, which have been the gold standard antibiotics for *P. aeruginosa* infection [6, 7].

Nanotechnology focuses on the employment of molecular and atomic techniques to arrange materials at a nanoscale with remarkable physical, chemical, and biological properties [8]. Nanotechnology has a wide variety of applications such as food, electronics, pharmaceuticals, fuels, chemicals, polymers, and environmental health [9]. Nanoparticles pose high reactivity due to the high surface, small size, and surface chemistry [10, 11]. In addition, as a result of significant antibacterial effect and low toxicity on human cells of metal nanoparticles (MNPs) like silver (Ag), cerium (Ce), iron (Fe), selenium (Se), silicon (Si), titanium (Ti), and zinc (Zn), they attracted special attention [11–13]. Also, MNPs exhibited antimicrobial functionality against a variety of microbes [11, 13]. Amidst MNPs, SeNPs are one of the most extensively studied [14]. Se gains a special palace in the field of medicine [15]. Se incorporated in the structure of several enzymes like glutathione peroxidases (GPx), iodothyronine deiodinases, and thioredoxin reductase (TrxR) that play role in the process like antioxidation, detoxification, and metabolism [8, 15–17]. Various forms of selenium oxyanions such as selenide (SeII), selenite (SeIV), or selenate (SeVI) have toxicity on animals as well as human cell lines while the elemental form of selenium (SeO) under biological concentration (<400 μg/mL) did not show toxicity. Also, selenium exists in either organic form like selenocysteine (Secys) and selenomethionine (Semet) or inorganic form including selenate (SeO_4^{2-}), selenide (Se^{2-}), and selenite (SeO_3^{2-}) [18]. SeNPs demonstrated remarkable antimicrobial activity against *Candida albicans* [19], *Streptococcus mutans* [20], *Staphylococcus aureus* [21, 22]. Moreover, the biological characteristics of SeNPs including antibacterial, antiviral, and antioxidant activity that possess low toxicity on human cells have presented an area for research in nanotechnology [8, 14].

Various physiochemical methods have been applied to synthesize SeNPs [16]. On the other hand, the synthesis of NPs by employing microorganisms and plants provides new, clean, safe, and eco-friendly techniques [8, 10, 17, 23]. Furthermore, the biosynthesized NPs generally capped via cellular metabolites that are related to the microorganisms [9, 24].

Different bacteria in the aquatic environments as well as terrestrial, either in selenium-rich or selenium-free soils, have the capability to reduce selenite/selenate oxyanions. As a result of the continuous introduction of industrial and urban swage, halophilic microorganisms face toxic metals, including Se which result in enduring toxic ions and changing them to wither less poisonous or nanoparticles [18, 25, 26]. Although the potential of salt-tolerant bacteria for reduction of selenite or selenate and other anions in high salt concentrations has been relatively less explored [16], existing in these severe environments like high-salt concentration needs cell components that make them typical microorganisms [9]. Lately, lots of researchers concentrate on halophilic microorganisms due to afford novel biomolecules [27]. Therefore, halophilic microorganisms have the capacity to be applied in biotechnological procedures including biopolymers, hydrolytic enzymes, biosurfactants, biofuels, and bioremediation [9]. Therefore, this study aimed to employ halophilic strain to synthesize SeNPs and determine their antibacterial properties on *P. aeruginosa*.

2. Material and Methods

2.1. Materials. Sodium Selenite was purchased from Sigma Aldrich. All other chemicals used in this study were of analytic grade.

2.2. Isolation and Characterization of the Bacterial Strains

2.2.1. Pseudomonas aeruginosa. In this study, 2 different strains of *P. aeruginosa* were used. These strains are *P. aeruginosa* ATCC 9027 and *P. aeruginosa* isolated from ulcers. Strains obtained from the University of Tehran have been molecularly identified.

For the PCR method, the primers were 9F and 1541R, and their sequences have been shown in Table 1. In addition, BLAST was performed via NCBI data bank.

2.3. Halophilic Bacteria

2.3.1. Isolation. The halophilic microorganism was isolated from Hoz-e Soltan area which is a salt lake located in Qom, Iran. In addition, to culture halophilic bacteria, salt water 10% (SW) is used.

2.3.2. DNA Extraction. In the first stage of extraction, remove some of the colony and add it to 200 μl of Tris-ethylene diamine tetra acetic acid buffer and then vortex it to dissolve completely. The contents of this buffer cause DNA to separate and also act as a chelator, leading to the loosening

Table 1: Primers sequencing was used for PCR assay to characterize the strains that isolated from wounds.

Primers	Sequence
9F	5′-AAG AGT TTG ATC ATG GCT CAG-3′
1541R	5′-AAG GAG GTG ATC CAG CCG CA-3′

of the wall. In the second step, 20 μl of 1 mg/ml lysozyme was added and incubated for 37 minutes at 37°C. This lysozyme breaks down the bacterial wall. In the third step, 100 μl of 1% SDS solution is added and reheated in the incubator for 30 minutes to dissolve the fat in the cell wall and eliminate it. In the fourth step, add 100 μl of 5 mM NaCl and leave the solution at 65°C for 30 minutes. Salt breaks down DNA-bound proteins more easily. In the fifth step, the same volume of chloroform solution was added and the solution was centrifuged at 11500 rpm for 15 minutes to form 3 layers. Separate the supernatant or top layer. The fifth step is performed three times, and in the sixth step, isopropanol (cold isopropanol) is added to the solution and refrigerated for 24 hours. In the seventh step, after 24 hours, centrifuge at 11500 rpm for 15 minutes and then remove the supernatant. In the eighth step, 70% ethanol is used, which is added in the amount of 200–300 microns per liter, dissolved in ethanol using blows, and in the next step, for 15 minutes centrifuge at 11500 rpm. In the tenth step, discard the topical solution, which was ethanol, so that no ethanol remains in the vial. In the last step, 200 microliters of TE buffer were used to store the extracted DNA.

2.3.3. PCR. To perform PCR on the DNA extracted in a vial, we add the materials in Table 2 in order and place them in the PCR machine. It is also a DNA-free vial, which we test as a negative control. The primers used with the desired sequences are also listed in Table 3.

2.3.4. 16srRNA Sequencing. The sample was sent to the Pishgam Company along with the 16SrRNA primer for sequencing. After sequencing the amplified fragment of the 16SrRNA gene, the isolated strain was compared with other sequences in the gene bank at the NCBI site using Blast software [28].

2.3.5. Phylogenic Tree. In this section, the similarity of the desired strain with similar strains was first obtained. For this purpose, the sequence edited by ChromasPro software is compared with the sequences registered in NCBI and EZTAXON databases. The sequence was then saved in the desired FASTA format to draw the phylogenetic tree. In the next steps, phylogenetic analysis of the desired strain and similar strains was performed using Mega7 software. Maximum liquidity method was also used to draw the phylogenetic tree of this strain. The validity of the branches in the drawn tree was checked using bootstrap analysis algorithm and sampled 1000 times.

Table 2: Materials required for PCR.

Materials	Amount (total volume = 30μ)
DDW	12
Forward primer	1
Reverse primer	1
Extracted DNA	1
Master mix	15

Table 3: Primers sequencing to employ for PCR experiments to characterize halophilic strain.

Primers	Sequencing
27F	5′-AGAGTTTGATCCTGGCTCAG-3′
1492R	5′-GGTTACCTTGTTACGACTT-3′

2.4. Biosynthesis of SeNPs

2.4.1. Intracellular. For intracellular synthesis, 200 ml of SW10% was inoculated with the isolated strain and sodium selenite (final concentration of 5 mM) which was filtrated through 0.2-micron filters. The mixture was incubated at 40°C for 24 hours. Simultaneously, uninoculated SW10% with sodium selenite was employed as a control [9].

2.4.2. Extracellular. For this method, the bacterium was first inoculated in 200 ml of sterile SW10% culture medium and then incubated at 150 rpm for 48 hours in an incubator shaker. In the second step, the inoculated culture medium was centrifuged at 4000 rpm for 20 minutes, and the supernatant was separated. The obtained supernatant was then passed through a 0.22-micron filter, and in the next step, 5 mM filtered sodium selenite was added to it. The mixture was placed in an autoclave for 20 minutes at a pressure of 1 atmosphere at 121°C [9].

2.5. Purification of SeNPs. To purify SeNPs, the bacterial cells were harvested by centrifuge at 4000 rpm for 20 minutes. Then, the lysis buffer (SDS1% and lysozyme) was added to the harvested cells. In addition, this mixture is sonicated for 20 minutes. 1-Octanol was added to this mixture and incubated overnight at 4°C. Sediments were collected and washed through chloroform, absolute ethanol, ethanol 70%, and distilled water, respectively [9].

2.6. Characterization of SeNPs

2.6.1. Visual Observation. Changing the yellow color of the culture media through visual observation confirmed the generation of SeNPs [9].

2.6.2. UV-Visible Spectroscopy. SeNPs were dissolved in distilled water through sonication (20 min, 110 W). 1 mL of dispersed NPs was added to the covet. Then, the absorbance of SeNPs between 200 and 800 nm was noted by Shimadzu

UV-1601, and also for the blank, distilled water is used. In addition, sodium selenite dissolved in water is used as a control [9].

2.6.3. Dynamic Light Scattering (DLS). 1 mL of dissolved SeNPs in water was sent to the Central Laboratory at Tehran University for measuring the particle size and distribution with utilizing HORIBA.

2.6.4. X-Ray Diffraction (XRD). This assay determined crystal shape of SeNPs [9]. Radiation source was recorded by the XRD method (40 kV/40 mA, 10° to 80°, a step size of 0.04 of seconds) [29].

2.6.5. Fourier Transform Infrared Spectroscopy (FTIR). To identify functional chemical groups on the exterior area of SeNPs [9], this assay was performed. The sample was tested using Tensor 27 via KBr pellet method as described by Nakamoto [30] in the range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1} [9].

2.6.6. Scanning Electron Microscopy. The SeNPs were fixed and stabilized for 2 hours in 2% of glutaraldehyde at 27°C, then it is dehydrated, and also coated with gold. The fixed SeNPs were observed via MIRA3 to identify the morphological characteristics of SeNPs [31].

2.7. Antibacterial Activity

2.7.1. Well Diffusion Assay. Two wells on the culture medium were produced by pipet Pasteur. Then, new culture of bacteria was prepared by Sterile swab. 0.0932 gr of SeNPs was dispersed in a 500 mL water. 100 μ of final concentration was loaded in one of the wells. For the second well that is considered as a standard well, 100 μ of culture media without bacteria and SeNPs were added.

2.7.2. Antibiotic Susceptibility. Susceptibility of isolated and standard strains to the antibiotic including Clindamycin, Nitrocefin, Sulfadiazine/trimethoprim, Sulphamethoxazole/trimethoprim, Ampicillin/sulbactam, Temocillin, Penicillin, Ceftazidime, Amphotericin B, Gentamicin, Ciprofloxacin, Pipemidic acid/tazobactam, Co-trimoxazole, Amikacin, Imipenem, Tetracycline, and Lincomycin was evaluated via the disk diffusion approach according to the Clinical and Laboratory Standards Institute (CLSI). To perform this experiment, a new culture of the strain was prepared, and antibiotic disks were added to each plate and incubated for 24 hours at 30oC.

3. Result

3.1. Characterization of Isolated Strains

3.1.1. Pseudomonas aeruginosa. Microscopic observation represented gram-negative and bacillus morphology of the strain. In addition, the result of the molecular assay has been presented in the table below (Table 4).

3.2. Halophilic Bacteria

3.2.1. 16SrRNA Sequencing. The result demonstrated that the isolated strain showed 98/30% similarity to the *Halomonas eurihalina*. This is the first report on the biosynthesis of SeNPs by *H. eurihalina*. In addition, the result has been shown in Table 5.

3.2.2. Phylogenic Tree. Moreover, with the sequence, the phylogenic tree was conducted via MEGA 7 which is shown in Figure 1.

3.2.3. Biosynthesis of SeNPs. This halophilic strain is able to produce the SeNPs via intracellular approach. In the succeeding parts, biosynthesized SeNPs were prepared for further analysis.

3.3. Characterization of SeNPs

3.3.1. Visual Observation. Color transition of culture media from to brick-red represented the formation of SeNPs which is shown in Figure 2.

3.3.2. UV-Visible Spectroscopy. For the primary confirmation, UV-Vis spectrophotometry was conducted. The sharp absorption peak has been seen at 275 nm that is possibly associated with the surface plasmon vibrations. However, no sharp peak is seen for the control sample (Figure 3).

3.3.3. Dynamic Light Scattering (DLS). Biosynthesized NPs size was within 200–400 nm, and also, the average size (Z-average) was 260 nm. Moreover, the polydispersity index was 0.2 (Figure 4).

3.3.4. X-Ray Diffraction (XRD). In Figure 5, on the 2θ axis of the diagram, there has been 5 peaks that belong to the 23.2°, 29.5°, 45.5°, 55.5°, and 59.8° corresponding to the (100), (101), (111), (113), and (202). In addition, this assay represented great peaks all over the whole spectrum ranging 20 to 80.

3.3.5. Fourier Transform Infrared Spectroscopy (FTIR). The first and second peaks, which are between 626.21 and 681.88 cm^{-1}, are related to C-Cl bond vibration. The third peak with wavelength cm^{-1} is 865.61 related to C-O. The fourth peak with wavelength cm^{-1} is 996.92, and the other peak with wavelength cm^{-1} is 1393.16 due to the presence of C-H bond. The 1637.60 cm^{-1} wavelength corresponding to the sixth peak is the first type of hope due to the presence of carbonyl amide bonds of proteins. The wavelength of 2175.12 cm^{-1}, which is the seventh peak, is due to the vibrations of alkynes (carbon-carbon triple bond). Also, the eighth peak with a wavelength of 2917.78 cm^{-1} is associated

Table 4: Different properties of wound strain.

Strain name	Sequence length	Microorganism	Accessibility code	Similarity
Wound sp.	739	*Pseudomonas aeruginosa* MLTBM2	MT646431.1	99/19

Table 5: Different properties of characterized halophilic strain.

Strain name	Sequence length	Microorganism	Accessibility code	Similarity
H98	780	*Halomonas eurihalina*	L42620	98/30

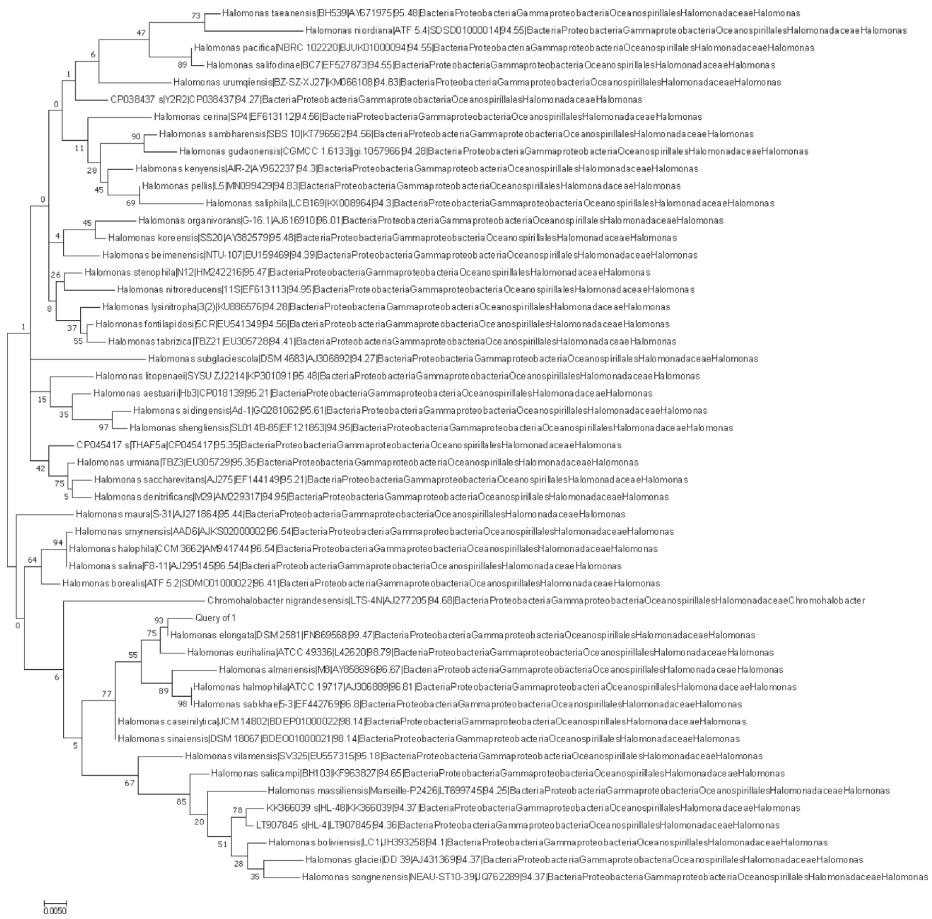


Figure 1: Phylogenic tree conducted by MEGA7. Query 1 represented the isolated strain that is the most similar to *Halomonas eurihalina*.

with asymmetric tensile vibrations of the C-H groups. The last peak related to the wavelength 3237.12 cm^{-1} which can be related to the O-H groups. These moieties inhibit the aggregation of SeNPs and make them more stable. All results have been shown in Figure 6.

3.3.6. Scanning Electron Microscopy. The results showed the dispersion of particles in size and also show that the biosynthetic nanoparticles have spherical surface (Figure 7).

3.3.7. Well Diffusion. The result showed 2.5 cm inhibitory zone diameter for standard strain and 2 cm for a strain isolated from wound. Also, the obtained results have been represented in the picture below (Figure 8).

3.3.8. Antibiotic Susceptibility. Based on susceptibility to disks, strains were divided into two groups sensitive and resistant. In this study, collected strains that were isolated of wounds and urinary were resistant to all types of antibiotic disks. In addition, respiratory strains were sensitive to Amikacin and Pipemidic acid/tazobactam and resistant to the other antibiotics, and also, the standard strain was sensitive to Co-trimoxazole, Amikacin, Temocillin, and Pipemidic acid/tazobactam. Table 6 demonstrates the results.

4. Discussion

The complication of a bacterial infection within the burn wound is one of the highest challenges in the burn clinic. It may result in more severe disease states such as sepsis. The

Figure 2: Conversion of Selenium to elemental Selenium.

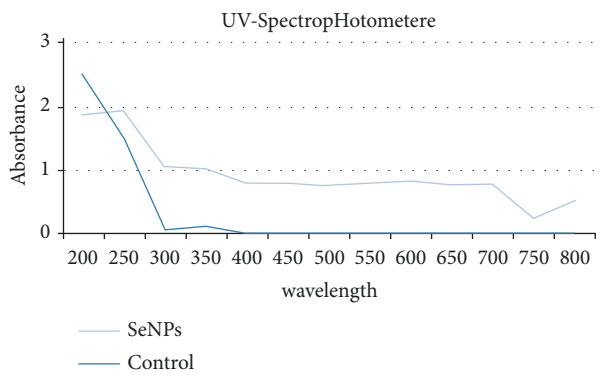


Figure 3: Control sample has not shown any absorption changes while at 275 nm SeNPs showed sharp peak.

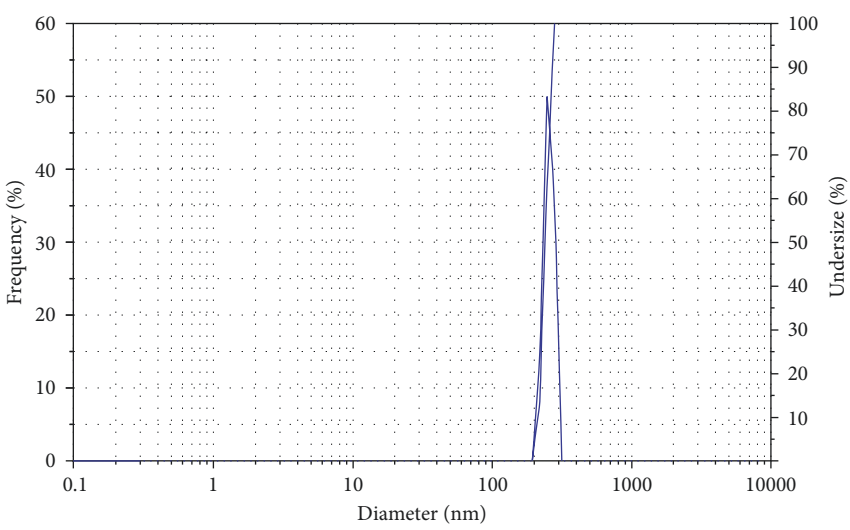


Figure 4: This picture shows the particles' size and distribution that 63% of NPs were 240 ± 49.

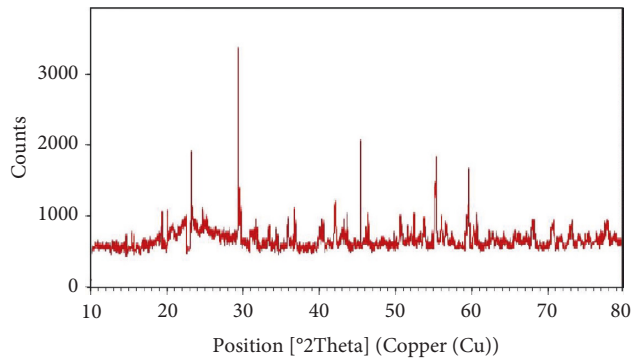


Figure 5: The results represented the crystalline nature of biosynthetic nanoparticles.

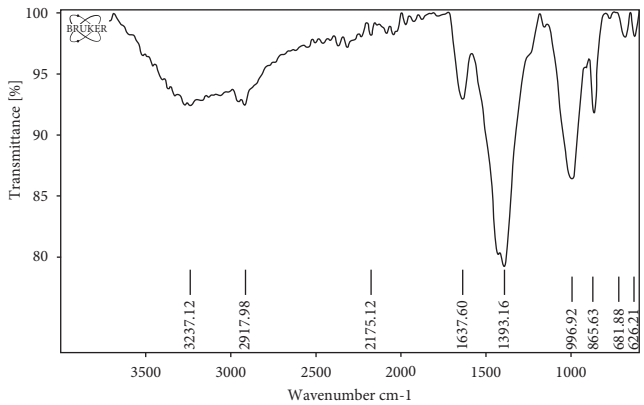
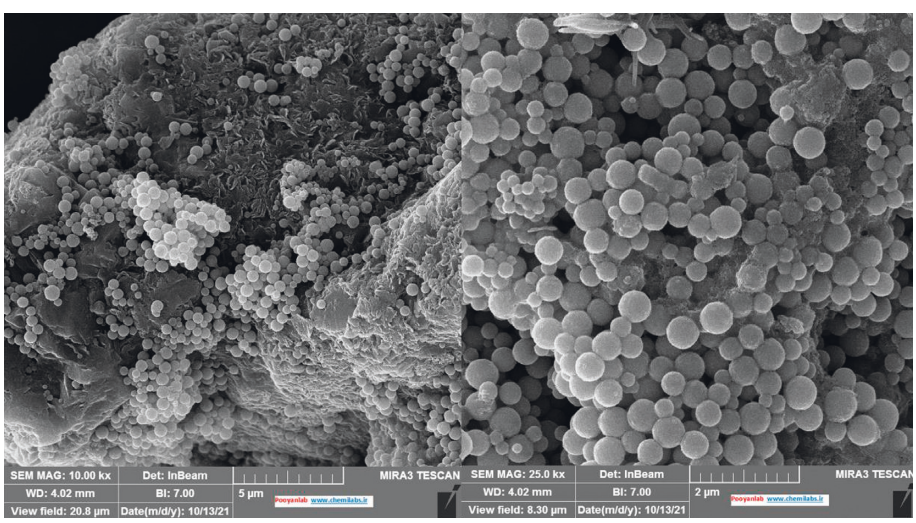


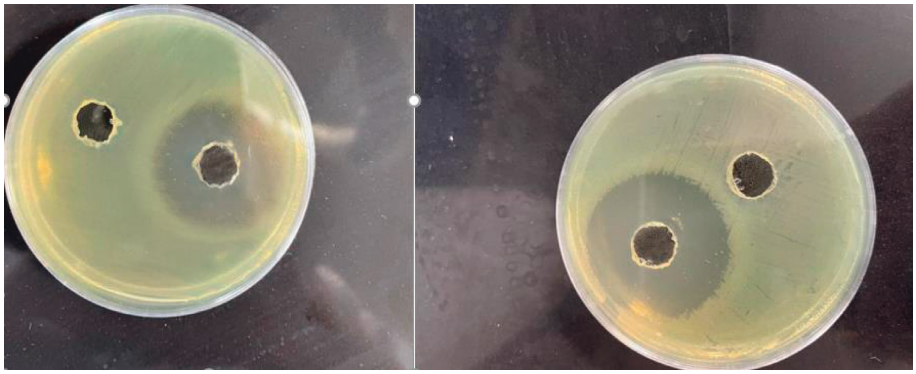
Figure 6: This chart is related to the results of FTIR test. The chart shows 9 peaks. Each of these peaks with the indicated wavelengths belongs to specific functional groups that have been involved in the synthesis or are known as nanoparticle stabilizers.



(a) (b)

Figure 7: SEM analysis results. (a) The right figure is with a magnification of 10,000; (b) The left figure is a biosynthetic nanoparticle with a magnification of 25,000.

challenge imposed by resistant microorganisms is further escalated by the scarcity of new antimicrobials, particularly antibiofilm agents in the treatment of burn wound infections. Various animal models are used for recapitulating the hallmarks of clinical burn wound infection and assisting to develop new antimicrobial treatments. The pigs, rats, and mice are the three most common models of burn wound infection *in vivo*. The best substitute for human skin is the porcine model. However, high costs and ethical implications hinder their usages for screening vast libraries of



(a) (b)

Figure 8: (a) Standard strain (left picture) showed more susceptibility to biosynthesized SeNPs; (b) also, the right picture is isolated strain.

antimicrobials. Mice present the highest range of advantages to characterize aspects of the host's reaction to burn infection, in terms of genetic mutants. However, they are often restricted to acute (less than 48 h postinoculation) burn wound infection prior to the fast onset of sepsis and death, possibly owing to requiring for injecting the higher bacterial inoculums under the burn eschar for inducing the burn infection. Formulating the pathogens in a biopolymer matrix is also included for driving the infection to a more chronic biofilm-like state [32].

Several studies demonstrated that the cells at the wound edges of injured airway epithelia and the denuded basement membranes are more susceptible to binding by *P. aeruginosa*. More specifically, it has been shown that *P. aeruginosa* adheres more frequently to mucus and extruded/dead cells as well as basal and migrating flattened cells with lamellipodia. Amino-acid sensor-driven chemotaxis and flagella-driven swimming appear to be the mechanisms by which *P. aeruginosa* reaches the cells at the wound edge of airway epithelia. Finally, fibronectin, asialoGM1, and $\alpha 5\beta 1$integrin may mediate *P. aeruginosa* binding to the airway epithelial cells [5].

According to the results obtained from molecular identification of 16SrRNA sequence, the obtained strain is 98/30% of *H. eurihalina*, which is isolated from Lut desert and also has the ability to biosynthesize selenium nanoparticles intracellularly.

To confirm the synthesis of nanoparticles, first, qualitatively by changing the color of the culture medium from yellow to red brick color, which is due to the conversion of selenite to selenium as an element, over time, the sodium selenite in the culture medium changes color to red [15, 23]. This indicates the reduction of selenite to elemental selenium, and also, the distinct red color created indicates the resonance of the surface plasmons of selenium nanoparticles. Slightly, the maximum light absorption was at 250 nm. In a study conducted by Dr. Mohammad Amoozgar and colleagues, the light absorption of selenium nanoparticles in the UV-Vis method was 294 nm [18]. In another paper, a group of scientists synthesized selenium nanoparticles by microorganisms isolated from different parts of Saudi Arabia, with the selected strain having an absorption peak of 290 nm [33]. Another experiment was performed in 2015 on the synthesis of selenium nanoparticles by the bacterium Ralstonia eutropha. The absorption peak for this synthetic nanoparticle is shown at 270 nm [34]. Various studies prove the resonance peak of synthetic selenium nanoparticles around 200–300 nm. However, this absorption peak also depends on various factors such as particle size, shape, material composition, and isolated environment of the desired strain [35]. In addition, there is a great deal of research on the formation of selenium nanoparticles, which indicates different absorption peaks for the presence of selenium nanoparticles [36].

The size of biosynthetic nanoparticles measured by the DLS test in this experiment was 260 nm on average, and the amplitude of the absorption peak in UV-Vis test also proves the dispersion of nanoparticle size. In addition, the dispersion index or PI was 0.2. In another experiment performed by Srivastava et al. on the synthesis of selenium

Table 6: Susceptibility of standard strain and wound strain to various antibiotic disks.

ATCC 9027	Wound sp.	
R	R	NI300
R	R	SAM20
R	R	IMI10
R	R	CIPR5
S	R	CTR30
S	R	TEM30
R	R	TS25
S	NT	AK30
R	R	GM10
S	R	PI + TZ
R	R	SXT
R	R	AMB
R	R	CAZ
R	R	P
R	R	DA
R	R	L
S	R	TE

Ni300: nitrocefin; SAM20: ampicillin/sulbactam; IMI10: imipenem; CIPR5: ciprofloxacin; CTR30: co-trimoxazole; TEM30: Temocillin; TS25: sulfadiazine/trimethoprim; AK30: amikacin GM10: gentamicin; PI + TZ: piperacillin/tazobactam; SXT: sulphamethoxazole/trimethoprim; AMB: amphotericin B; CAZ: ceftazidime; P: penicillin; DA: clindamycin; L: lincomycin; TE: tetracycline (R: resistance; S: sensitive; NT: not tested).

nanoparticles by a bacterium, the average particle size was 70.9 nm [34]. Another experiment synthesizing selenium nanoparticles from a bacterial source showed that the particles were 170–160 nm in size [37]. It has been proven that when the scattering indices are less than 0.5, the nanoparticle stability is higher and the nanoparticle aggregation rate is lower [38].

In addition, according to research, selenium nanoparticles synthesized by bacteria are coated by various biomolecules or macromolecules such as proteins and polysaccharides. In fact, the reduction of metal salts to metal nanoparticles can be caused by carbohydrates and proteins. FTIR analysis can provide useful semiquantitative results in relation to the presence of biological compounds in the nanoparticle coating layers and their associated contents by the vibration of the functional groups (proteins and carbohydrates) of the nanoparticles [39]. The test results prove the presence of proteins and polysaccharides.

According to the results of the XRD experiment, the synthesized selenium nanoparticles have a natural crystalline nature. In another study, XRD results show that synthetic selenium nanoparticles are crystalline, which is a natural form [40]. However, in other experiments, the results showed that the synthetic selenium nanoparticles did not have any specific crystalline form, and amorph was seen [28, 41].

In addition, based on the results of the SEM test, synthetic nanoparticles are dispersed in size. This dispersion may be due to the accumulation of nanoparticles. Also, based on the morphology shown in the results, it is observed that the obtained particles have a spherical surface. In an experiment conducted by a group of researchers in 2018, synthetic selenium nanoparticles also had a spherical shape [28, 41]. In 2016, the results of SEM analysis also showed that the biosynthetic SeNPs by *P. aeruginosa* have a spherical surface [42].

5. Conclusion

Regarding the result of various analyses, it is concluded the biosynthetic SeNPs by halophilic bacteria represented suitable characteristics which possess antibacterial properties against the most common pathogenic bacteria. Hence, it is concluded that biosynthetic SeNPs can be considered as an alternative candidate for traditional antibiotics to lessen the side effect of employing antibiotics and also increasing the efficiency of therapeutics. On the other hand, further analysis and in vivo tests are in demand to find out the various aspects of employing these NPs.

Data Availability

All the data generated or analyzed during this study are included in this published article, and also, the data sets analyzed to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Effect of Collagen/Ibuprofen Hydrogel in Wound Healing: An In Vivo Study

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Background and Aim. Wound healing is a complicated physiological process to preserve skin integrity after injuries and includes the proliferative phase, hemostasis/inflammatory phase, and remodeling through extracellular, intercellular, and intracellular components synchronization. The study aimed to examine human placental collagen/ibuprofen's effect on wound healing in an animal model. *Materials and Methods*. The cell viability test was performed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. In this animal study, three circular excisions were made in the back of each of the 12 rats and injected with the following groups: collagen/ibuprofen, group 1; collagen, group 2; and unfilled as the control group, group 3 for two times. The healing procedures were explored via calculating wound contraction percentage after taking photographs on days 7 and 14. Also, histopathological assessments were conducted on days 7 and 14. *Results*. The MTT results showed no significant cytotoxicity, and macroscopic results demonstrated that the percentage of wound contraction was 85%, 79%, and 41% in the collagen/ibuprofen, collagen, and control groups on 14th day, respectively. Histopathological assessments showed that hair follicles number, fibroblasts content, and angiogenesis in the collagen/ibuprofen were remarkably more significant than collagen and control groups. *Conclusion*. The collagen/ibuprofen group had significant outcomes compared with collagen and the control groups in wound healing and wound contraction. Collagen/ibuprofen hydrogel can be a potential hydrogel in accelerating wound healing.

1. Introduction

Skin wounds are prevalent injuries that generally occur via different causes involving metabolic diseases such as diabetes mellitus and thermal or physicochemical damages. Skin wounds have the potential to induce morbidity along with imbalance of function, disability, and elevated pain [1]. The statistical analysis estimated that about 5.6 million people in the United States are impacted via wound lesions, and treatment costs over 50 billion dollars each year. Also, skin wounds can be categorized into chronic and acute wounds. Acute wounds usually heal themselves sufficiently and smoothly [2]. Contrarily, circumstances such as related disorders, inadequate nutrition, the extensive extension of the wound, age, and impairment of the skin regeneration process lead to chronic wounds [3]. Wound treatment concluded with wound debridement, pressure off-loading, and infection management with topical antibiotics and

topical antiseptics also revascularization, which are the main principles of therapeutic wound approaches [4]. There are different advances in debridement such as surgical processes, biosurgery, wet to dry dressing, preparations of enzymes, polysaccharide beads or dextranomer polysaccharide paste, and hydrogels. When wound dressing alters the wound from moist to dry, they provide nonselective debridement, hence cleaning the wound with necrotic tissue removal. It is supposed that wounds be investigated at each dressing change. Moisture-retentive dressing concluding films, hydrocolloids, hydrofibres, alginates, and hydrogels are helpful in diverse clinical surroundings [5]. One of the critical clinical problems is wound care therapy, which possesses several procedures containing inflammation, hemostasis, proliferation, angiogenesis, connective tissue remodeling, and wound strength recovery. Regeneration and spontaneous growth generally restore the integrity of damaged tissue in the wound healing process. The endogenous release of chemokines, cytokines, and growth factors is crucial for restoring wound tissue integrity that mediates the interaction between fibroblasts, keratinocytes, neutrophils, endothelial cells, and macrophages [6]. Pain management is a significant burden with chronic wounds in patients. Also, cells release pain neuropeptides responding to a broad range of stimuli, and responses to pain stress can be detrimental to amelioration. Neuropeptides generally induce monocytes, leukocytes, and other immunoreactive cells to discharge proinflammatory cytokines [7]. An imbalance of proinflammatory cytokines impacts wound healing negatively, subscribing to related inflammatory disorders and leading to the destruction of tissues. Multiple advances to prevent acute pain and inflammation in wound care have been assessed. Also, ibuprofen-loaded materials with controlled-release actions have been utilized to prevent immoderate inflammation through the early phases of healing, attenuate pain, and stimulate the repairing of tissues in the duration of later phases [8]. Ibuprofen has been classified in a nonsteroidal anti-inflammatory drug (NSAID) class to treat acute pain, inflammatory, and degenerative diseases by prohibiting cyclooxygenases (COX) pathways. Ibuprofen is biotransformed rapidly due to its short half-life and must be used rapidly to preserve effective plasma concentration. Nevertheless, multiple doses of ibuprofen can hinder compliance and decrease clinical efficacy [9]. Moreover, ibuprofen's sustained and controlled release systems can improve the conditions of therapeutic burdens by stimuli-responsive carriers providing demanding drug release [10]. Similarly, with injectable hydrogels, temperature-inducible, localized drug delivery systems may modulate drug release and alleviate nonspecific drug distribution to healthy organs and tissues. Likewise, they can support inhibition of multiple drug-using and increase patients' compliance [11]. Collagen provides integrity of tissue structures and cells that generally recognize the most presented protein in the skin. Arranged myofibroblasts and fibroblasts in the fibril forms of tissues synthesize skin collagen regularly under shear stresses and tensile [12]. One of the forms of collagen is type I collagen, which exists in the fasciae, tendons, dermis, and components of essential scar tissue. Collagen is used to administer in the synthesization of skin substitutes that are also delivered in transmembrane proteins composition, fibril surfaces, or beaded structures [13]. Therefore, the purpose of the study was to investigate the effect of collagen and ibuprofen hydrogel on wound healing in the in vivo test and also assess the histopathological changes and the percentage of wound contraction.

2. Materials and Methods

2.1. Synthesis of Hydrogel. Collagen was extracted according to the protocol [14]. In brief, the human placenta was derived from the abdomen via a general surgeon. Afterward, the placenta was washed (distilled water, 4°C) and then chopped 1-2 cm on ice. Sodium hydroxide was used for removing the noncollagenous proteins (1N, refreshed every 2 h, stirred for 6 h). Then, extra fat was eliminated by butyl alcohol (10%, 24 h, filtered with cheesecloth). The precipitate of 20% collagen was acquired using NaCl (pH = 7, 3M, centrifuged 5000 g for 30 min). The remaining was dissolved in 2% acetic acid, transferred to dialysis tubing, stirred in distilled water (5 days), and freeze-dried [14]. The harvested collagen was immersed in diluted hydrochloric acid (pH = 2, to obtain 1% (w/v) solution) [15]. Then, ibuprofen (-2-(4-isobutylphenyl) propionic acid; Arora Pharmaceuticals, Delhi, India) (25 wt%) was added to the collagen solution while stirring. The obtained homogeneous suspension was stored at 4°C.

2.2. Cell Viability Test. The MTT (methyl thiazolyl tetrazolium) test was done directly according to the ISO 10993–5 standards. The cell culture plates maintain the negative control without cytotoxicity. MTT assay was conducted to assess the cytotoxicity of the hydrogel. Hence, 20000 adipose-derived stem cells (ADSCs) were added to the culture medium (50 μL), and 20000 fibroblasts were added to the culture medium (50 μL) as the negative control without cytotoxicity, separately which are containing serum and cultured on the hydrogel. The culture medium was added to each well after 3 hours and was removed. Afterward, MTT solution (100 μL, 0.5 mg/mL) was added to each well. The supernatant solution was eliminated after 4 hours. Then, DMSO (100 μl) was added to dissolve the formed purple formazan crystals. The solution optical density was detected at 570 nanometers wavelength in an ELISA reader (Convergent ELReader 96X). Findings were shown as a percentage (control value = 100.00%). Also, the assays were displayed in triplicate to acquire accurate outcomes. The cell viability was investigated via the following equation:

$$\textbf{Cell viability}\,(\%) = \frac{\text{Value of therapy sample}}{\text{Value of sample control}} \times 100. \quad (1)$$

2.3. In Vivo Test. Twelve male Wistar rats (180–200 g and 3-4 weeks) were allocated in standard conditions (20–25°C, 65–75% humidity) under the supervision of a veterinarian. General anesthesia was induced by intramuscular injection of 2% xylazine (10 mg/kg) and 10% ketamine (50 mg/kg).

Afterward, three circular excisions (8 mm in diameter) in the back of each of the 12 rats were made using a punch on day 7 and day 14. The defective sites were injected as follows: collagen/ibuprofen (group 1), collagen (group 2), and unfilled control group (group 3). The extracted collagen (3 mg/ml) was mixed with 40 mg/ml of ibuprofen, and then, the mix was injected over the wound.

2.4. Macroscopic Analysis. In macroscopic analysis, wound contraction pictures were obtained of the excision wounds in all three groups using a digital camera 7 and 14 days after the intervention. The Image J software investigated the wound contraction pictures concerning the wound contraction percentage. The wound contraction percentage was calculated using the following equation .

$$\text{Wound contraction}\,(\%) = \frac{\text{Initial wound size} - \text{specific day wound size}}{\text{Initial wound size}} \times 100. \quad (2)$$

2.5. Microscopic Analysis. The rats were sacrificed after 7 and 14 days. The samples (each group with 1 cm area) were fixed in 10% formalin for 48 h. Then, the samples were dehydrated in a graded series of 80%–100% ethanol solution. All the specimens were located in paraffin, cut into a 5 μm section, and then stained with hematoxylin and eosin staining. The samples were evaluated and photographed using a light microscope. The histological investigations, including the numerical density of angiogenesis, hair follicles numbers, and fibroblasts, comprise a morphological description of at least three sections under a light microscope.

2.6. Statistical Analysis. The nonparametric Friedman test was used to compare the groups at $P < 0.05$ level of significance.

3. Results

3.1. Cytotoxicity Test. This cell viability investigation was performed at 24 and 48 hours. In this test, cells cultured on conventional culture dishes were considered control. According to Figure 1, the results showed that the survival of cells was less than the control group in 24 hours and overtime; an increasing trend of survival is observed, which may indicate that cells resumed their reproduction after tolerating a shock on the first day and went through an upward trend. Furthermore, the cell survival rate in 48 hours reached about 98% in both groups (Figure 1).

3.2. Macroscopic Results. The rats were sacrificed and photographed at 7 and 14 days, and wound contraction was evaluated. Figure 2 shows the macroscopic perspective of the wounds. The outcomes showed that the wound healing rate was higher in the collagen/ibuprofen group than in other groups. Wound contraction and healing in the collagen/ibuprofen group were more significant than in collagen and control groups. Wound contraction was 40%, 25%, and 17% in the collagen/ibuprofen, collagen, and control groups on day 7, respectively. On day 14, wound healing and contraction were more remarkable than day 7, and collagen/ibuprofen could significantly enhance wound healing. Wound contraction was higher in the collagen/ibuprofen group than in collagen and control groups. Wound contraction was 85%, 79%, and 41% in the collagen/ibuprofen, collagen, and control groups on day 14, respectively (Figures 2 and 3).

3.3. Microscopic Results. Histopathological investigations reported proper wound healing in the collagen/ibuprofen group on day 7. The fibroblasts relocated into the wound site, and hair follicles were detected on day 14, which showed the crucial role of synthetic hydrogel in the improvement of wound healing and stimulation of other factors migration that possess practical functions in wound healing progression on day 14 (Figures 4 and 5). Hair follicles, blood vessels, and fibroblasts were discovered in the collagen/ibuprofen group in 7 days compared with other groups, indicating that this synthetic compound (collagen/ibuprofen) can play a fundamental role in the enhancement of wound healing. The procedure remained and expanded by day 14, which implied the positive response of cutaneous tissue to the synthetic hydrogel and its crucial part in the increment of wound healing (Figures 4 and 5). The rate of wound healing was higher in the collagen/ibuprofen group during 14 days compared to 7 days. The cells were transferred to the wound site and promoted angiogenesis on day 14 more than day 7. Wound healing was observed with collagen sheets and slight migration of cells into the wound site in the collagen/ibuprofen group; however, higher migration of cells and further angiogenesis were detected on day 14 in the collagen/ibuprofen group. Both hair follicles and angiogenesis were considerable in collagen/ibuprofen and collagen groups, but the number of fibroblasts in the collagen/ibuprofen group was higher than the collagen group. Wound healing in the collagen group began from the peripheral areas of the wound during 7 days and was enhanced by day 14, based on slight migration of cells to the wound area and increased angiogenesis.

On the contrary, wound healing was unremarkable in the control group, and negligible healing was detected under the layer of connective tissue. The wound healing rate was significantly higher on day 14 than on day 7 in the control group. The number of blood vessels, hair follicles, and fibroblasts (vital factors in wound restoration) was drastically lower in the collagen and control groups on days 7 and 14 compared to the collagen/ibuprofen group.

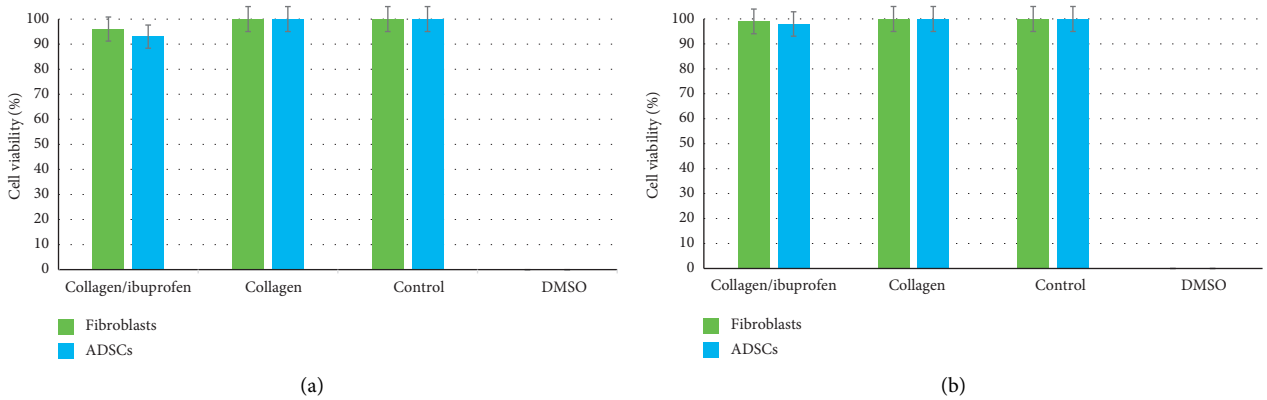

Figure 1: Cell viability of fibroblasts and ADSCs in control, collagen, and collagen/ibuprofen groups at 24 (a) and 48 (b) hours ($P < 0.05$).

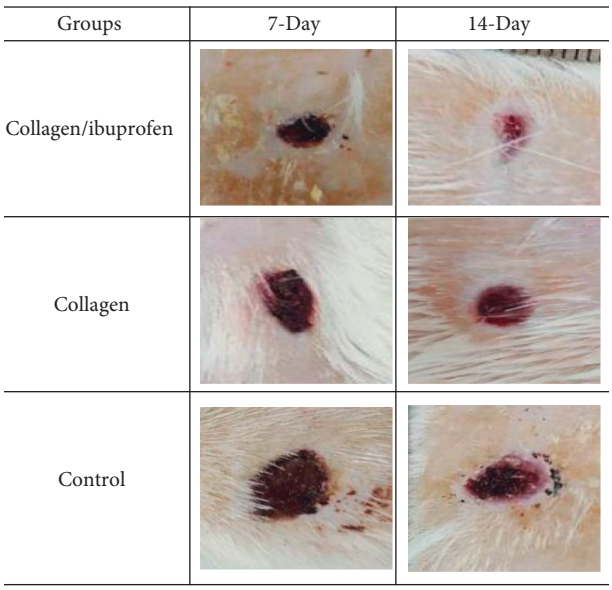

Figure 2: Macroscopic investigations of control, collagen, and collagen/ibuprofen groups on days 7 and 14.

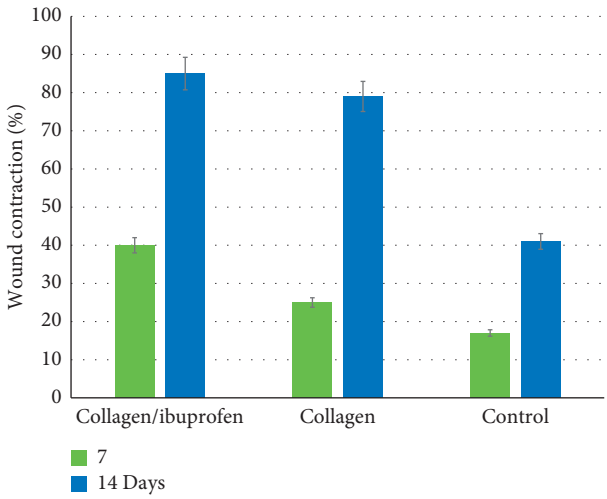

Figure 3: Wound contraction of collagen/ibuprofen, collagen, and control groups on days 7 and 14 ($P < 0.05$).

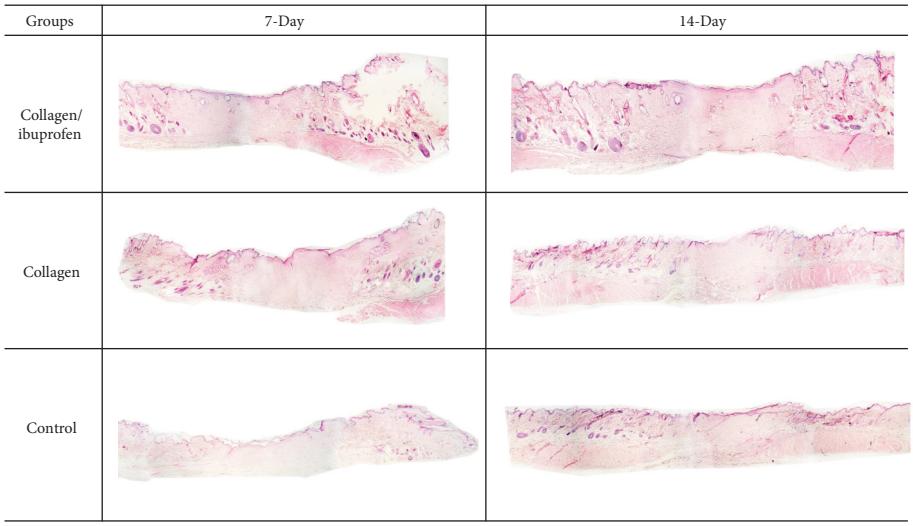

Figure 4: Histopathological analysis of collagen/ibuprofen, collagen, and control groups on days 7 and 14 with H&E staining (×40).

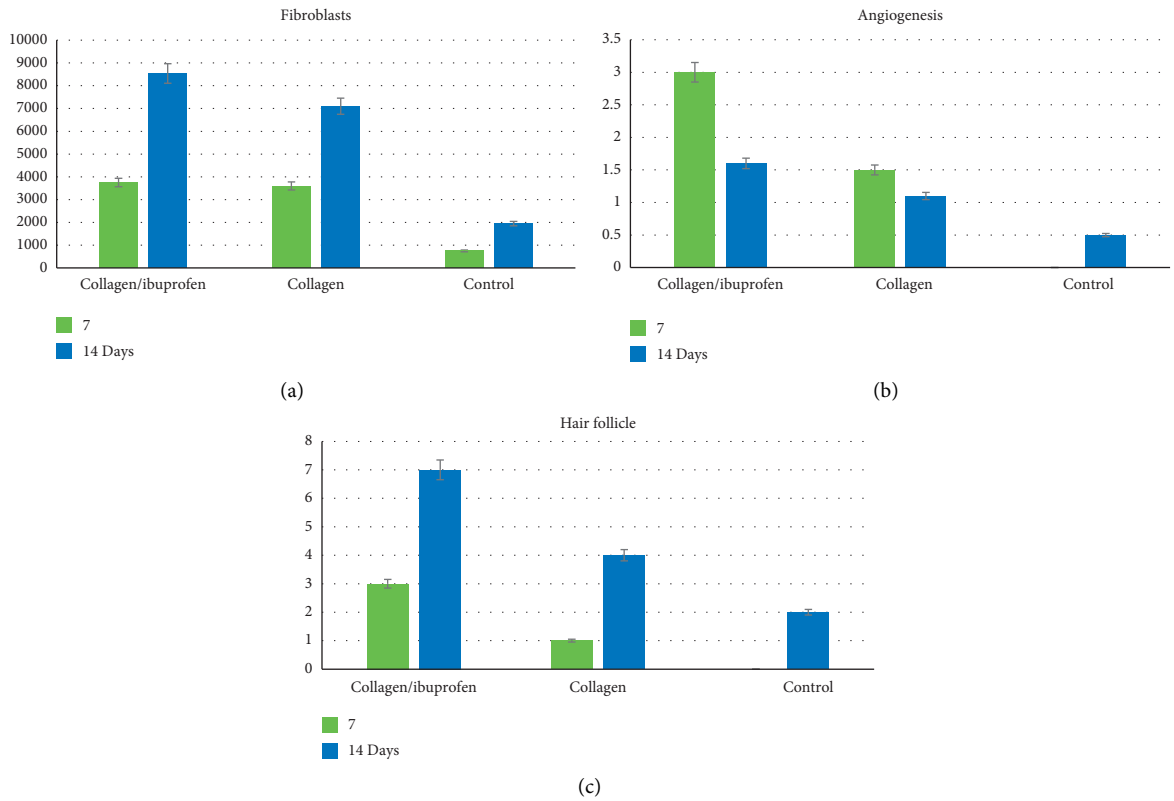

Figure 5: Fibroblasts (a), angiogenesis (b), and hair follicles (c) of collagen/ibuprofen, collagen, and control groups on days 7 and 14 ($P < 0.05$).

4. Discussion

The progression of functional therapy for increasing angiogenesis and wound healing, especially for complicated or nonhealing wounds, demands the ability of the compound to work in the challenging environment of insufficient wound healing. Additionally, several components have shown significant promise in increasing angiogenesis and wound healing in animal wound healing models. Previously, various wound healing therapeutic approaches that have been prosperous in preclinical investigations eventually deteriorate to present advantages to patients when examined in clinical trials [16, 17]. Hydrogels are broadly administered for repairing and regenerating damaged tissues, principally because of their characteristics, such as permeability, viscoelastic properties, porosity, biocompatibility, and

biodegradability. Wound healing experiments reported that hydrogels showed induced dermal regeneration and anti-inflammatory characteristics. For example, microspheres of gelatin integrating ginsenoside Rg1 (a compound derived from *Panax ginseng*) were loaded in chitosan/collagen scaffolds. Therefore, the dressing was usually administered to regenerate the damaged tissues of the skin because of the effect of the bioactive compounds [18, 19]. The inflammatory reactions are a series of well-coordinated cellular occurrences causing wound repair that can perform both deleterious and beneficial roles in repairing cutaneous layers. NSAIDs are abundantly used to manage inflammation because of their antipyretic, analgesic, anti-inflammatory, and thrombotic properties because of inhibition of cyclooxygenases 1 and 2 [20, 21]. Also, NSAIDs such as ibuprofen are the first-line drugs for treating osteoarthritis, soft tissue injuries, gout, pain, and inflammatory diseases with an estimated administration of more than 30 million per day. Recent findings have revealed that the combination of anti-inflammatory factors such as diclofenac and dexamethasone can decrease levels of prostaglandin E, edema, and histopathological damage when used after the manifestations of first clinical symptoms. Diclofenac presents to affect via inhibition of cyclooxygenases 1 and 2, hence inhibiting prostaglandin synthesis. Likewise, clobetasol inhibits phospholipase A2 and cyclooxygenase 2 activity by suppressing the glucocorticoid receptor and inhibiting cytokines production and altering the population of cells at the inflammation area. Also, the effectiveness of combination therapy improved due to different mechanisms of diclofenac and clobetasol [22]. Ibuprofen would have had the most significant impact on healing in the early alleviation period in the duration of acute inflammation reactions to defect and injury [23]. Connizo et al. have explored that the early use of ibuprofen in the postoperative duration devastated tendon healing [24]. In contrast, delayed use did not affect the improvement of the tendon. The findings of Connizo et al. also supposed that ibuprofen administration has the most significant restorative effect in early postoperative duration (between 2 and 4 weeks) [24, 25]. Korat and Kapupara showed that local infiltration of the surgical wounds with low doses of ibuprofen, levobupivacaine, and epinephrine affects the sutured muscle wound in postoperative pain. The surgical wound was infiltrated with 50 μL solution comprised of 8 mg/mL epinephrine, 0.3% w/v levobupivacaine, and 2 mg/mL ibuprofen and in contrast to infiltration of water over the sutured muscle wound before closing the skin. The related outcomes to the study revealed that local infiltration of the surgical wound with epinephrine, levobupivacaine, and ibuprofen combination has efficient functions in the surgical wound healing and postoperative pain through reducing pain, improvement of angiogenesis, and strength of tensile in the rat model study [26]. Li et al. studied that the chitosan/collagen gel combination supplemented with a cell-penetrating peptide gel had a fantastic rate of healing and acceleration in treatment via increasing granulation tissue formation, stimulating angiogenesis, and enhancing deposition of collagen in wound tissue. In contrast, negligible cytotoxicity of gel was reported in the histopathological analysis [27].

This study demonstrated that wound healing was significantly enhanced in the collagen/ibuprofen group than collagen and control groups. Wound contraction was 40% in collagen/ibuprofen groups, while this rate was 25% in the collagen and 17% in the control group on day 7. Contrarily, the rate of wound contraction was more significant on day 14. Wound healing in collagen/ibuprofen groups was more remarkable than in the collagen and control groups. The collagen group showed higher wound healing and contraction than the control group. On day 7, wound healing in the collagen/ibuprofen group was highly considerable; migration of fibroblasts to the wound site and observation of hair follicles at the healing site on day 14 presented that the synthetic hydrogel significantly enhanced the wound healing and promoted the migration of other factors involved in this process to the wound site. The rate of healing in the collagen/ibuprofen group on day 14 was higher than that on day 7 and characterized by the formation of collagen layers and migration of cells and angiogenesis. On day 14, the wound margins' closure was more excellent in the collagen/ibuprofen group. The healing in the control group was negligible on day 7 and was initiated beneath the connective tissue layer. The healing rate on day 14 was much higher than that on day 7 in the control group. Based on the outcomes, the collagen/ibuprofen synthetic hydrogel may be concluded to enhance wound healing during 14 days effectively. Microscopically, the collagen/ibuprofen synthetic hydrogel increased wound healing and induced the migration of factors responsible for the healing process, which presented the promising capacity of the synthetic hydrogel comprised of collagen and ibuprofen in acute wound healing.

Data Availability

The data used to support the findings of this study are included within the article and are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Review Article

Biomaterials in Guided Bone and Tissue Regenerations: An Update

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Purpose. Guided tissue reconstruction can be performed to restore the supporting structure of a previously lost tooth, which, in addition to maintaining beauty, preserves the function of the tooth in the patient. *Materials and Methods*. In this review, Scopus, PubMed, and MEDLINE databases were searched using the keywords "biocompatible materials," "membrane," "bone regeneration," "tissue reconstruction," and "dental biomaterials." Overall, 150 articles were reviewed, and finally, 107 articles published during 2000–2021 were included in the final paper. *Results*. Studies have been conducted on a variety of membranes in both clinical and experimental settings. The first half of this article explores the different kinds of membranes and diverse classes of biomaterials used in these procedures. Secondly, biomaterials are examined for their therapeutic uses such as growth factors, stem cells, and gene delivery vehicles. *Conclusion*. If a tooth has been extracted or if the gums have been infected with periodontal disease, guided bone regeneration procedures may be used to restore the lost bone. Recent years have seen a variety of approaches to regenerating these tissues. To prevent nonossifying cells from entering, membranes are heavily employed during guided rebuilding.

1. Introduction

Today, one of the most critical topics in periodontology is the repair of periodontal lesions and the reconstruction of lost jawbone through proliferating bone cells. A fundamental prerequisite for successful implant treatments is the adequate volume of hard bone tissue [1]. On the other hand, for the successful repair of damaged tissue, it is necessary to prevent the invasion of cells with high-speed migration to the damaged tissue and guide sedentary cells to the lesion site.

In recent years, tissue engineering using biocompatible polymers has introduced new methods for repairing damaged tissues [2]. Guided bone regeneration (GBR) and guided tissue regeneration (GTR) are the most important conventional reconstruction methods. The GBR is used to regenerate alveolar bone in toothless areas, and GTR is utilized to repair damaged periodontal tissue [3, 4]. In general, GTR and GBR are surgical techniques that use a porous polymer membrane to physically prevent the migration of undesirable tissues and cells to the lesion site [4]. As a result, a suitable space and substrate are provided for

repairing damaged tissue by proliferating the cells in question [5]. The GBR can maintain and strengthen the alveolar bulge, regenerate alveolar bone, correct contractions around the implant or fenestration, and regenerate the bone around the implant [6]. In contrast, GTR refers to periodontal ligament (PDL) regeneration, bone regeneration, and cementum around the tooth [7].

Although porous membranes in the GBR technique seem necessary, repairing and growing damaged bone require osteogenic cells, as well as osteoconductive and bone-inducing (osteoinductive) materials [8]. The ideal membrane used in guided bone tissue repair should have unique properties, such as good biocompatibility and functional stability over the required time. In addition, the membrane must maintain the space and biomechanical stability of the lesion area by filtering out the disturbing cells and tissues and protecting the newly formed tissue [9].

Generally, membranes used in GTR and GBR techniques may fall into the following categories: (1) bioabsorbable based on natural polymers, including collagen, chitosan, and gelatin, or synthetic polymers; (2) nonresorbable membranes, including expanded polytetrafluoroethylene, titanium-reinforced e-PTFE, and dense-PTFE [10]; and (3) metals and inorganic compounds considered as another group of guided membranes, which will be discussed in detail.

2. Materials and Methods

Based on the institutional regulations, this research was granted an exemption regarding approval since it was a literature review. A comprehensive search of the Scopus, PubMed, and MEDLINE databases was performed. All relevant articles published during 2000–2021 were obtained using the keywords "biocompatible materials," "membrane," "bone regeneration," "tissue reconstruction," and "dental biomaterials." Afterwards, articles were reviewed by titles and abstracts. The papers that were less relevant to the subject of study were excluded. The remaining full-text articles were evaluated, and those unrelated to the subject were removed. The filtered papers were further analyzed by the team of authors, and this review was structured.

3. Bioabsorbable

A significant advantage of this type of membrane is that it does not have to be removed by secondary surgery, that it has a better repair and healing ability, that it is biocompatible, and that it reduces the risk of inflammation and infection [11]. However, there are several limitations to these structures, the most important of which is the low ability to make a suitable space for new tissue growth and the high destruction rate. These structures are destroyed in the body much faster than expected and leave the damaged area before completing the tissue formation process [12, 13]. The two kinds of adsorbable membranes consist of natural polymers and synthetic polymers [14].

3.1. Natural Adsorbable Membranes. The application of natural polymers has expanded due to their properties in the GTR and GBR processes. In other words, inherent bioactivity and the ability to provide active sites for cell attachment are among the most important advantages of natural polymers over synthetic polymers [15]. However, some problems related to the intrinsic bioactivity of polymers, including strong immune responses, complications associated with the purification of these polymers, and the possibility of disease transmission, restrict the use of these polymers [16]. Table 1 summarizes the disadvantages and advantages of various types of membranes.

3.1.1. Collagen Membranes. Collagen is the hardest filament in the connective tissue. This membrane has significant advantages, such as good tissue integrity, fast vascularization, biodegradability without external reactions, weak immunogenicity, hemostatic property, biocompatibility, and wound healing capacity [42]. In addition, collagen is a chemotactic factor for fibroblasts and accelerates cell migration. Collagen membranes may secondarily increase tissue thickness as a result of enzymatic degradation and replacement by surrounding connective tissue [43]. Such properties have led to collagen-based membranes in GTR and GBR research. This type of membrane is degradable and adsorbable by enzymatic degradation, carried out by collagenases and proteases [44, 45]. Porcine collagen fiber types I and II are incorporated into Bio-Gide, one of the most commonly used commercial collagen membranes. Soft tissue invasion and development into a defect may be limited by the smooth surface of Bio-Gide, which may also act as a scaffold for the attachment of fibroblasts [46–50]. The rough, porous side of Bio-Gide functions as a framework for the migration and proliferation of blood vessels and bone cells [43, 46].

Disadvantages of collagen membranes have rapid biodegradability and reduced membrane capability in maintaining the space and biomechanical stability of the lesion area in wet conditions. The destruction time of these membranes is about 4–8 weeks, which is not enough for the full regeneration of bone tissue [51, 52]. To improve the mechanical properties and decrease the rate of deterioration of collagen membranes, several approaches have been investigated [42, 53]. There are a number of possibilities for treatment with UV light or chemical therapies with genipin (Gp) [54, 55].

Moreover, various physical, chemical, and biological crosslinking methods effectively strengthen the mechanical characteristics and augment the stability of collagen membranes against biodegradability. The most common chemical crosslinking agents are glutaraldehyde, 3-dimethyl-aminopropyl, carbodiimide, and polypoxy [45, 55]. Crosslinking is accompanied by pros and cons. Its advantage is improving the tensile strength of collagen and delaying the breakdown time [56]. Drawbacks entail limiting the use of membranes by creating potential toxic effects by the remaining crosslinking agent or the formation of byproducts during collagen degradation, which can lead to severe inflammation at the

Table 1: Advantages and disadvantages of various membranes.

Membrane type	Membrane materials	Advantage	Disadvantage	References
Natural polymers	Collagen, gelatin, chitosan	• High biocompatibility • Improved cellular Interaction •Hydrophilicity •Antibacterial effect •Cell/drug containing •Enhancement of wound healing •No surgical removal	• Lack of bioactivity • Rapid degradation rate •Low mechanical strength •Residual crosslinking agents •Possible disease transmission •Questionable barrier function •Hard to control biodegradation •Few studies	[17–27, 51, 52, 57, 60]
Synthetic polymers	PLA/PLGA, PCL, PEG	• Mechanical strength can be processed • Able to seed mesenchymal cells/growth factors •Favorable biocompatibility •Favorable barrier function •High reproducibility •Manageable biodegradability and mechanical properties •No surgical removal needed	• Slow degradation rate • Hydrophobic (PCL) •Low cell affinity •Poor cellular response •Not suitable for a drug delivery system •Acidic byproducts	[28–34]
Nonresorbable	e-PTFE, d-PTFE, TR-ePTFE	• Many studies demonstrate their success • Could be titanium-reinforced •Remain intact until removal •Easily fixed with titanium or resorbable tacks •Greater bone fill if the membrane is not exposed •Minimal tissue response if the membrane is not exposed •High chemical stability •High biocompatibility •High barrier function	• Require a second surgery for removal • Increase patient morbidity •If exposed, must be removed in most cases •Could be technique sensitive •Membrane exposure	[12, 35–38, 87]
Metals	Titanium, titanium alloy	• High biocompatibility • High barrier function •Mechanical strength, durability	• Surgical removal required • Expensive	[39–41, 91]

implant site [51, 57]. Crosslinking, in addition to reducing the rate of degradation, greatly diminishes tissue integration and angiogenesis and also affects the biocompatibility of the final structure. Increasing the percentage of crosslinker decreases the adhesion and proliferation of PDL fibroblasts and osteoblasts [58, 59]. Therefore, the success of the operation by collagen membrane, in addition to the source of the collagen, depends on the preparation and processing stages, including decellularization, sterilization, and crosslinking methods. The Gp and D-ribose, as two natural safe and nontoxic compounds, have been proposed to raise the mechanical strength of collagen and diminish its degradation rate [51, 60, 61].

3.1.2. Gelatin-Based Membrane. Gelatin is a protein that is soluble and is made from collagen. Improved cell adhesion, suitable biocompatibility, reasonable price, and flexibility of this protein have made it a favorable biomaterial for tissue engineering, GTR, and GBR [62, 63]. Protein membranes have poor mechanical properties, and they degrade rapidly. A method for improving stability and mechanical properties is crosslinking glutaraldehyde and N-hydroxyl succinamide using heat treatment [64]. However, crosslinking can reduce the modulus of elasticity under humid conditions, even though it improves the tensile properties of gelatin fiber membranes. Because of this, gelatin can sometimes be used alone in GTR and GBR [65, 66].

3.1.3. Chitosan-Based Membranes. Studies showed that chitosan is well adapted to cells in vitro and facilitates bone regeneration at the site of rat skull lesions [67]. Chitosan is also a suitable membrane for GTR and GBR because of its reasonable price, high biocompatibility, good degradation rate, antibacterial properties, wound healing potential, and flexibility in humid environments. Chitosan membranes deteriorate at different rates depending on their molecular weight and manufacture method [68]. One of the ways to boost mechanical strength and reduce the rate of chitosan degradation is chemical crosslinking. Gp-crosslinked chitosan membranes produce fewer inflammatory reactions than glutaraldehyde-crosslinked membranes, accelerating lesion healing [69]. Chitosan-based electrospinning membranes crosslinked with Gp have a much lower degradation rate than nonlattice membranes. Due to the high cost of Gp and the toxicity of glutaraldehyde, ionic crosslinking with sodium tripolyphosphate has been proposed as an alternative for crosslinking [70].

3.2. Synthetic Adsorbable Membranes

3.2.1. PLA and PLGA. The PLA is among the most important and common synthetic polymers applied in the GTR and GBR processes. This polymer has high mechanical and biocompatible properties. To control the hydrophilicity of PLA and its degradation rate, its copolymers are synthesized based on lactide, caprolactone, and glycolide [71]. Polyglycolic acid (PLGA), in orthopedic applications, is an excellent alternative to PLA. Despite the biodegradability and nontoxicity of PLA and PLGA-based membranes, there are limitations to using these membranes. One of these problems is the possibility of inflammatory reactions and reactions to an external object in vivo when oligomers and acidic byproducts are released during degradation [72]. Their hardness is another problem that limits the medical applications of these membranes. One effective solution for the hardness is to add emollients, such as N-methyl-2-pyrrolidone (NMP). The NMP softens PLGA membranes, thereby accelerating bone regeneration and osteoblast growth [73].

3.2.2. Poly-Caprolactone (PCL). A low-cost polymer is also biocompatible and possesses excellent mechanical strength. Therefore, it has received much attention in bone tissue engineering. There are still few studies on PCL-based GTR membranes. One of the advantages of this polymer over PLA and PLGA polymers is that PCL degradation does not increase environment acidity [74]. The complete bioabsorption of the PCL membrane in the body takes about 3 years, which is long for use in the GBR and GTR methods. On the other hand, the hydrophobicity of its pure membranes reduces adhesion and cell proliferation. Consequently, it is always combined with other polymers or as a copolymer for medical applications [75, 76].

3.2.3. Polyethylene Glycol (PEG). This polymer is remarkably biodegradable and biocompatible, making it a suitable option for use in GTR and GBR membranes [77]. The main positive points of this polymer membrane are regulated biodegradation, manageability, processability, and the ability to encapsulate the medicine [75, 78]. In contrast, poor instability and high degradation rates produce a robust inflammatory response that affects the outcome of bone regeneration and leads to regenerated bone resorption [79, 80].

4. Nonresorbable

The first used membranes were nonresorbable and able to keep the bone lesion separate from other tissue cells for a long time. In these membranes, reoperation is often required to remove the membrane. The repair will not occur spontaneously if these membranes are exposed. In addition, there is a possibility of bacterial contamination of the exposed membrane resulting in infection [81].

4.1. Expanded Polytetrafluoroethylene (e-PTFE). The e-PTFE membranes are the first synthetic polymer used for GBR with cavities of 0.02–20 μm and are an excellent candidate to cover the defect opening around the implant and repair or maintain the bone around the implant in the area [82–85]. Due to its high chemical stability, this membrane is biologically considered the most stable polymer, which resists degradation caused by host tissues and does not lead to immunological reactions [80]. They also prevent bone and

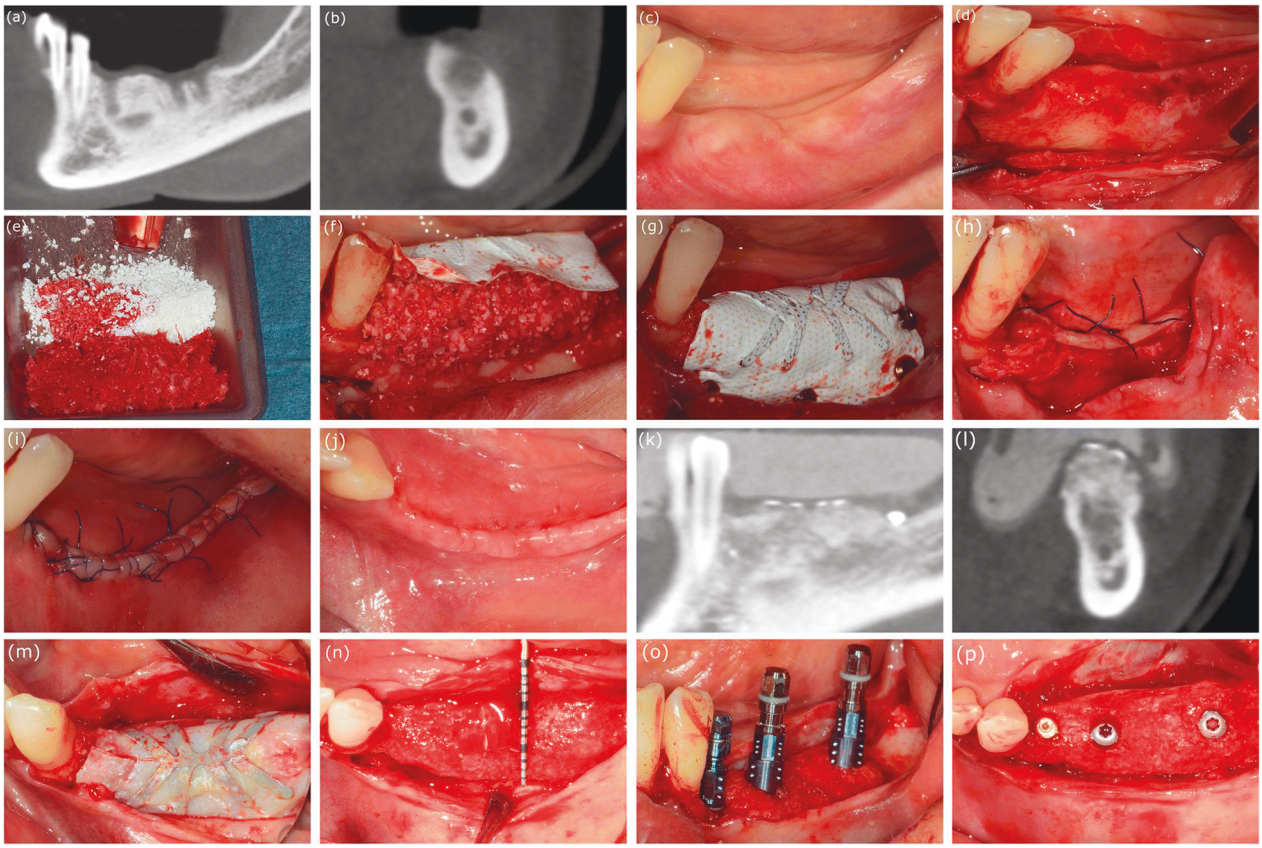

Figure 1: Bone augmentation with a titanium-reinforced d-PTFE membrane. (a) Primary CBCT scan, parasagittal section showing the horizontal and vertical bone defect. (b) Primary CBCT scan, frontal section showing the horizontal and vertical bone defect. (c) Clinical photograph of the edentulous mandible. (d) Flap preparation with the split-thickness method. (e) Bovine-derived xenograft (Geistlich Bio-Oss) and particulate autogenous bone graft. (f) Adaptation of nonresorbable titanium-reinforced d-PTFE membrane (Osteogenics Cytoplast) over the graft. (g) Fixing the membrane with titanium pins. (h) Suturing. (i) Closure of the mucosal layer with horizontal mattress and noninterrupted sutures. (j) Healing two weeks post-op. (k) Postoperative CBCT scan after nine months: parasagittal section. (l) Postoperative CBCT scan after nine months: frontal section. (m) Reentry for removing the membrane. (n) Good quantity of bone tissue for implant placement. (o) Guided implant surgery (Straumann bone level). (p) Final positioning of implants through a prosthetically driven technique [92].

other connective tissue cells from migrating to the lesion [86]. As a result, bone marrow cells, which migrate less rapidly, are more likely to be present at the lesion site. Despite the expansion of tetrafluoroethylene membranes, osteogenesis without interfering with other tissues leads to repairing damaged bone tissue in an average period of three months. In contrast, osteogenesis occurs incompletely in control samples without membrane because of connective tissue interference [87].

4.2. Dense Polytetrafluoroethylene (D-PTFE). The porosity of the e-PTFE membrane can allow germs to migrate. This problem was addressed through the creation of d-PTFE membranes with pores smaller than 0.3 m. Clinical studies have found that d-PTFE does not generate bacterial colonies as rapidly as e-PTFE [88, 89]. The d-PTFE membranes used in the mouth cavity may prevent bacteria from passing through, but they do allow oxygen to enter and tiny molecules to pass through. On the other hand, e-PTFE membranes have larger holes and can interact more strongly with soft tissue. This results in membrane separation being significantly more challenging and requiring deeper incisions following hard tissue regeneration. As there is no development or attachment to soft tissues, d-PTFE membrane separation is straightforward [90].

4.3. Titanium-Reinforced Expanded Polytetrafluoroethylene (TR-ePTFE). Flexible titanium mesh-reinforced polytetrafluoroethylene membranes allow the membrane to be ductile to fit the shape and size of the defect in the desired area and provide adequate structural stability in bone defects around the implant [91]. Figure 1 demonstrates an implant surgical procedure using a titanium-reinforced d-PTFE membrane [92]. Clinical and experimental studies on nonabsorbable membranes have shown good therapeutic results in GTR and GBR applications. However, one of the most important disadvantages of these membranes is the need for secondary surgery to remove them after bone growth. In addition to raising the cost to patients, this sometimes leads to the loss of a part of the regenerated tissue. Stiffness and rigidity are

other weaknesses of nonabsorbable membranes that screws are used in most cases to make them more stable [56].

4.4. Metals. A frequent medical material used in maxillofacial surgery and orthopedics, titanium, is highly biocompatible, strong, corrosion-resistant, dense, and light [93]. In addition to bone grafts, titanium mesh has been shown to improve the localized deficiency of the alveolar ridge prior to or after implant placement [94, 95]. Studies have shown that titanium causes less lasting inflammation compared with PTFE [96]. When considering chronic inflammation connected to titanium micro/nanoparticles, there is a well-established mechanism by which metal nanoparticles cause inflammation via their immunomodulatory properties, which mostly act at the macrophage level [97, 98]. A number of factors increase with increasing oxidative stress, including DNA damage, protein carbonylation, lipid peroxidation, and superoxide dismutase activity, while catalase and total glutathione are all declining. In addition, they result in macrophage activation that is aberrant, which is associated with an increase in inflammation and a decrease in innate immunity [99, 100]. Ti nanoparticles were investigated in vitro to determine their effects on MSC physiology, ROS generation, and phenotyping of osteogenic and adipogenic cells. Activating PKC beta, which is implicated in MSC commitment to adipocyte lineage, also causes ROS production, and metal particles are not degradable, so ROS production causes abnormal neutrophil recruitment. The bone regeneration function of VEGF is reduced by a genetic abnormality. This imbalance affects the osteogenic commitment [101]. Another used metal is cobalt-chromium (CoCr), which has suitable mechanical properties, but less biocompatibility than titanium. One study found that placing a CoCr membrane on the tibial defect of a rabbit created enough space for bone regeneration [102].

5. Inorganic Components

Calcium sulfate (CaS) is a substance used to form significant GBR membranes because it is osteoconductive, biocompatible, and bioresorbable [103, 104]. It is possible to make solid substances with relatively stable and less absorbable crystals by hydrating CaS hemihydrate powder [105, 106]. It is a calcium phosphate of the hydroxyapatite type. HA is frequently used in bone applications due to its resemblance to bone minerals and biocompatibility. The biocompatibility and osteoconductivity of HA make it a common material used in bone treatments. Membranes with strong mechanical properties can withstand soft tissue static pressure while allowing bone to regenerate more readily [107]. When combined with nonabsorbable and absorbable membranes, membranes that are mixed with HA have been shown to enhance osteoblast-like cell activity in vitro [108–113].

6. Biomaterial-Based Delivery

6.1. Growth Factor Delivery. Signal molecules such as growth factors control how cells grow and develop. Several cell functions are affected by growth factors, including proliferation, migration, and extracellular matrix (ECM) formation. Some of them play a role in cell differentiation [114]. Growth factors include VEGFs, FGFs, PTHs, PDGFs, and IGFs. When cells begin to repair themselves, PDGF is released, which is chemotactic and mitogenic. Angiogenesis is induced by FGF, and cells such as fibroblasts, periodontal ligaments, osteoblasts, and endothelium are proliferated and differentiated by this growth factor [115]. Similarly, FGF signaling contributes to the development of the cranial skeleton [116]. Figure 2 illustrates a method developed to deliver growth factors to scaffolds [117]. The combined use of PDGF and grafts results in bone regeneration of up to 5 mm, whereas grafts alone result in bone regeneration of about 1-2 mm. Therefore, it is expected that growth factors will enhance the results of grafting materials [114]. To maintain skeletal health and growth, IGFs are necessary [118]. The mesenchyme also plays a role in vascularization, proliferation, and bone formation in the skull and maxilla [119]. Periodontal lesions are treated with plasma-rich plasma (PRP) derived from one's own centrifuged blood. This can be used alone or with grafting materials. Although there is insufficient evidence to suggest a relation between PRP and maxillary sinus floor elevation, it appears to have benefits in treating periodontal lesions [120, 121]. The PDGFs are known to contribute to bone repair, wound healing, and regeneration following trauma or infection by stimulating osteoblastic progenitor cells to multiply [122]. There is histological evidence that the enamel matrix derivative (EMD), Emdogain, can regenerate periodontal lesions due to its high amelogenin content and small amounts of enamel and other proteins. Additionally, angular periodontal lesions can be treated with it [123, 124]. In addition, EMD contains growth factors that stimulate the production of growth factors, such as bone morphogenetic proteins (BMPs), and enhance angiogenesis. In addition, EMD contains growth factors that encourage angiogenesis and enhance bone morphogenetic proteins (BMPs). In several studies, the combination of demineralized freeze-dried bone allograft (DFDBA) with EMD has been studied and found to be successful in regenerating periodontal lesions [125, 126].

Bone matrix proteins, or BMPs, play an important role in bone development at all stages. In addition to their contribution to the formation of the neural crest, rhBMP-7 and rhBMP-2 are also involved in the development of the teeth, lips, palates, and facial primordia, as well as creating soft and hard calluses [116, 127]. In the process of creating it, orthopedics, periodontics, and dentistry were considered. By interacting with the ECM, growth factors are stabilized and maintained in vivo. To achieve steady and local release of one or several GFs, selecting the right biomaterial delivery method is crucial [128]. To capture GFs physically or chemically, sponges, micro/nanoparticles, nanofiber membranes, and hydrogels are being used as delivery vehicles [129]. In addition to maintaining release kinetics, the GFs in the biomaterial provide a porous scaffold that facilitates internal bone growth [118, 130]. The following examples demonstrate two effective strategies. According to Jung, recombinant BMP-2 has the potential to enhance and expedite gingival resorption in humans, as well as for a wide

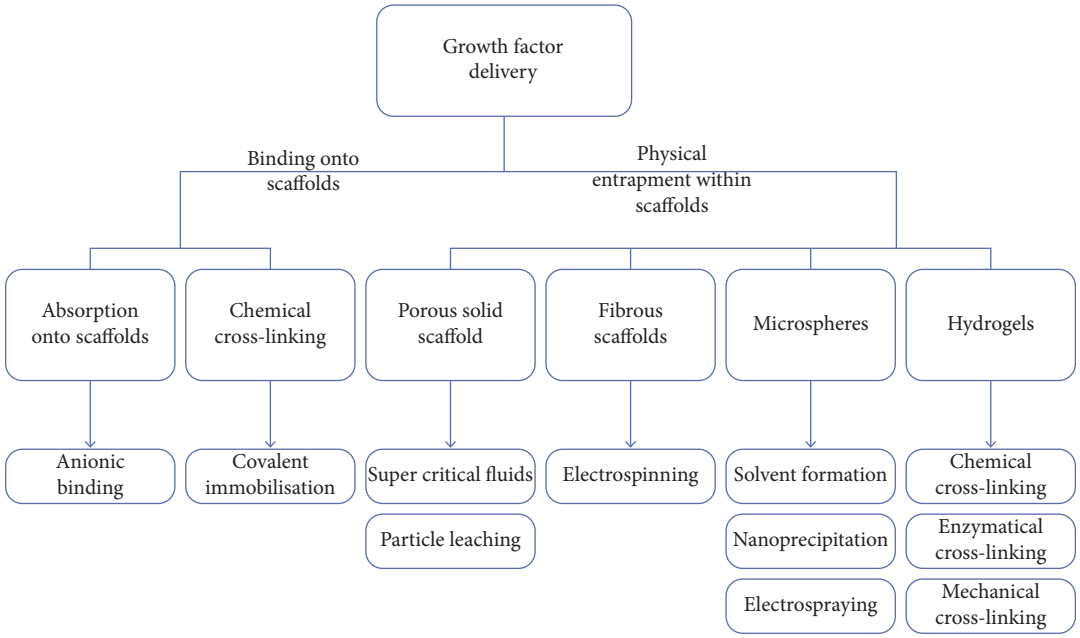

Figure 2: Current methods for fabrication of growth factor-loaded scaffolds [117].

range of bone defects. These include (1) rhBMP-2 injected into absorbable collagen for sinus lift and local augmentation of alveolar ridges, (2) OP-1 putty and rhBMP-7 for nonbonded fractures, and (3) TCP injected with rhPDGF for periodontal tissue [131]. Another study revealed that human periodontal ligament cells developed early osteoblasts when recombinant BMP-7 was added [132]. In bone development, bone production, and homeostasis, BMPs function in tandem with transforming growth factor-beta (TGF-beta) by activating signaling pathways [133].

6.2. Stem Cell Delivery Vehicles. Biomaterials can also be used for the delivery of stem cells. Embedded cells can be connected and developed using biomaterials rather than native ECM, avoiding ankylosis. In many cases, biomaterials can be altered to activate the cellular processes required for tissue regeneration and to prevent a host immune response [134, 135]. Hydrogels allow stem cells to be delivered minimally invasively to the face and jaw to repair deformities and disorders of the skull. Adipose tissue, dental pulp placenta, bone marrow, and limbus adult progenitor and stem cells were utilized in clinical studies [136]. Only a few commercially accessible materials based on mesenchymal stem cells are available for clinical uses such as DBM, Trinity Evolution Matrix™, Map3™, Osteocel Plus®, and AlloStem® [137]. A report by Soe and others indicated that mesenchymal stem cells existed in the periodontal ligament [138]. Overall, stem cell administration via biomaterials appears to help regenerate and restructure the oral cavity. Nevertheless, further research is required to evaluate the safety of the medication and its effectiveness in the long run [139].

6.3. Gene Delivery. The short half-lives of many growth factors used in tissue engineering restrict their availability at the right time and in the right amount. Recently, genes have been used to stimulate cells to create growth factors. Biomaterials have been preferred over other virus vector systems for their safety and ease of modification and mutagenesis [118]. A study by Giannobile et al. successfully transferred BMP-7 and PDGF genes to fibroblast, cementoblasts, and other periodontal cells [140]. Transplantation of cells expressing these genes into periodontal wounds stimulated bone and cement regeneration in rats [141]. Using this technology, periodontal repair simulations can be simulated, although additional studies on the efficiency and safety of the method are necessary [142].

6.4. Scaffold and Cell-Free Technologies. To diagnose prognostic human illness, scientists continually develop new models for diseases, detect early indicators, and experiment with new treatments. Humans are capable of curing a variety of illnesses through multipotent stem cells. Cells of the mesenchymal stem cell line may differentiate into a variety of cell types and function as paracrine glands that secrete endogenous chemicals that affect the immune response and aid tissue repair [143]. It is difficult to safely use MSC banking for rapid regenerative applications due to the unique challenges specific to each MSC type, including technical, legal, and ethical issues. EVs are a group of small vesicles that are released from a different cell types and heterogeneous cultures by budding from the plasma membrane. There are many different vesicles that are seen in EVs, such as exosomes, shed vesicles, nanoparticles, and apoptotic bodies [144]. The terms ectosome, microparticle, and nanoparticle refer to single vesicles released directly from the plasma membrane. The origin of exosomes has been implicated in numerous studies; however, reliable information regarding EV origins is often lacking. Nano- or microvesicles are classified according to their size. The secretome of a cell includes extracellular vesicles. The major

components of exosomes are nucleic acids, proteins, cytokines, enzymes, and misfolded proteins [143]. In light of their many features to be utilized for diagnosing, prognosticating, and therapeutic purposes, EVs are regarded as new and clever theranostic instruments [144]. Studies have examined how MSC-derived exosomes can be used in the regeneration of kidney, liver, hearts, and neurological damage in a variety of model illnesses and conditions. Scientists have recently focused on ESC-MSCs. Several methods were employed for examining the components in the conditioned medium, including multidimensional protein identification technologies, gene microarrays, and cytokine antibody arrays [144]. Based on computational analysis of the acquired data, computational analysis of the gene products involved in immune response and tissue differentiation was predicted [145]. According to Zhang et al., exosomes produced from human embryonic MSCs can be used to heal cartilage and subchondral bones and can therefore be considered a "cell-free" therapeutic method for osteochondral disorders [146].

6.5. Other Potential Biomaterials. These days, smart material research focuses on allotropic materials that have unique properties including medicinal applications that make them valuable components. Smart two-dimensional materials are being studied mainly due to their unique allotropic properties, which have shown potential in a number of applications. Two-dimensional materials that are nanometer scalable can be used to enhance the interaction between cells and human tissue. Biocompatible and bioactive interfaces are needed between cells and biomaterials. Recent studies have demonstrated the effectiveness of MoS2, WSe2, and h-BN as biomedical device fabrication materials [147]. It has been consistently found that two-dimensional materials exhibit distinct physical, chemical, electrical, and optical properties. The antimicrobial and physical properties of graphene have been widely reported in recent years, but the material also has certain limitations. In the coming years, black phosphorene (BP) could replace graphene as a potential material for biomedical applications due to its material structure and biological properties. In the same way as other 2D materials, BP may be used to make colorimetric and fluorescence detectors and biosensors. Furthermore, BP produces nontoxic byproducts in the body after in vivo biodegradation. The property of this material could be beneficial for pharmaceuticals, prosthetic coatings, and scaffolds. Because BP is a low cytotoxic agent, there are no local effects [147].

Inzana and others focused their 2014 research on creating calcium phosphate scaffolds using low-temperature 3D printing. Biolinking these scaffolds to bone tissue chemicals is possible because these scaffolds showed good cytocompatibility and osteoconductive properties [148]. There is already BP in bone, but in tiny amounts, comprising 1% (slightly more than 660 grams) of the total body weight [149]. A biolinkage may use this characteristic to combine with chemicals that enhance osteoconduction [148]. The role of calcium and phosphate in bone healing is well known in tissue engineering. The researchers focused on bioglass-based scaffolds made from BP nanosheets. A variety of scaffolds can be created using bio-printing. A 3D printing technique that uses biomaterials that have been doped or coated with BP might be a viable way of improving osteosarcoma therapy [150]. Wang et al. created experimental scaffolds that mimicked medullary bone by creating reticular damage to promote and enhance cellular attachment and colonization. Nanosheets of BP (200–400 nm) were applied to the scaffolds to bind them safely and effectively. Based on results from in vitro testing, the coated scaffolds were very capable of promoting bone formation due to increased cell proliferation on their surfaces, which may have been explained by their unique shape. The BP-BG scaffolds have been demonstrated to be more effective at treating post-oncological bone abnormalities in a mouse model of osteosarcoma [150]. The BP coating was also applied to hydrogels. In particular, gels were created by photo-reticulation of gelatin containing methacrylamide with ultraviolet light (GelMA). GelMA and BP were coated with arginine and poly(ester amide). A functionalized hydrogel facilitated bone development. The hydrogel's compression modulus and biodegradability time were measured in vitro, where BP submerged in mimicked physiological fluids produced a positive response. In addition, BP-based hydrogels promoted the proliferation of human dental pulp stem cells (hDPSCs) when differentiation into osteoblasts occurred. Based on the results of this study, BP-coated hydrogels may be suitable for use in dentistry if they provide the optimal environment for hDPSCs [151]. Then, 2D boron sheets have been grown on Ag substrates to produce two-dimensional triangular structures known as borophene (BO). As with graphene, boreophene regularly exhibits anisotropic properties. Because of its simple 3D structure, borons are classified as metalloids because neither metals nor nonmetals can be formed from their structure. Several semiconductors are fabricated using this material. The metal properties of boron are more apparent when it is arranged in a two-dimensional structure. This is comparable to allotropes such as graphene [152]. A borophene ridge's form and size are determined by how strongly boron atoms bond together. Because of this, the surfaces of graphene and borophene are significantly different. Borophene is a polymorphous and anisotropic compound due to its structural feature [153]. The properties of borophene make it a fascinating material for biomedical applications. This material is known as the "chameleon" of biomedical materials because it exhibits a variety of chemistry and physical properties that are suitable for both medical devices and customized biomedicine, exhibiting a variety of behavior and existence in many phases.

7. Conclusion

To place a suitable implant, doctors have examined the tissue and bone repair to put a crown/root ratio that is ideal, as well as long-term stability of soft tissue. The epithelium is separated from the damaged tissue with the help of different kinds of degradable septic membranes in GTR and GBR

treatments. Because e-PTFE membranes are indestructible, additional surgery is required to remove them. They have the greatest flaw of all. Despite their biodegradability and cell adhesion, natural polymer-based membranes have poor mechanical strength and short degradation cycles. Synthetic polymer-based membranes can be controlled for their biodegradability and mechanical strength. They, however, have a lower biological activity than natural polymers. Additionally, their decomposition products might trigger inflammatory responses outside the body. Despite some drawbacks, the importance and irreplaceability of biodegradable polymers cannot be overstated in GTR and GBR procedures, while periodontal biomaterials have become increasingly popular over the past several decades, owing to their many benefits ranging from testing membranes to everyday use. Biomaterials will be determined by factors found in genes and stem cells that control cell growth. Furthermore, membrane ossification activity needs to be studied to maintain a balance between their mechanical and biological features.

Abbreviations

GTR: Guided tissue reconstruction/regeneration
PDL: Periodontal ligament
GBR: Guided bone regeneration
PLA: Polylactic acid
PLGA: Polyglycolic acid
NMP: N-methyl-2-pyrrolidone
PCL: Poly-caprolactone
FGF: Fibroblast growth factor
PEG: Polyethylene glycol
PTFE: Polytetrafluoroethylene
e-PTFE: Expanded polytetrafluoroethylene
d-PTFE: Dense polytetrafluoroethylene
TR-ePTFE: Titanium-reinforced expanded polytetrafluoroethylene
CoCr: Cobalt-chromium
CaS: Calcium sulfate
HA: Hydroxyapatite
PDGF: Platelet-derived growth factor
PTH: Parathyroid hormone
BMP: Bone morphogenetic proteins
PRP: Platelet-rich plasma
IGF: Insulin-like growth factor
EMD: Enamel matrix derivative
ECM: Extracellular matrix
VEGF: Vascular endothelial growth factor
DFDBA: Demineralized freeze-dried bone allograft
GFs: Growth factors
DBM: Demineralized bone matrix
Gp: Genipin
TGF-β: Transforming growth factor-beta
MSCs: Mesenchymal stem cells
EVs: Extracellular vesicles
ESC-MSCs: Human embryonic stem cell-derived MSCs
2D: Two-dimensional
BP: Black phosphorene
GelMA: Methacrylamide gelatin
U-Arg-PEAs: Arginine and poly (ester amide)
hDPSCs: Human dental pulp stem cells.

Data Availability

The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Comparison of the Effect of Extracted Bacteriocin and Lytic Bacteriophage on the Expression of Biofilm Associated Genes in *Streptococcus mutans*

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Today, various biological approaches are used to combat dental plaque biofilms. In the current study, we aimed to compare the effect of extracted bacteriocin and lytic bacteriophage on the expression of biofilm associated genes in *Streptococcus mutans*. *Streptococcus mutans* was isolated from plaques of decayed dental and the existence of their gtfs genes was confirmed by PCR. For bacteriocin extraction, at first two probiotic lactobacilli were isolated from traditional foods, then bacteriocin was extracted by partial purification method, and SDS-PAGE was used for estimation of its molecular weight. The previously extracted lytic bacteriophage from raw urban sewage was used against *Streptococcus mutans*. Finally, the effect of isolated bacteriocin and bacteriophage on gtfs genes expression level was measured by real-time PCR. Out of 81 dental plaque samples, 32 (39.5%) *Streptococcus mutans* strains were isolated. The frequency of genes was as follows: gtfD32 (100%), gtfB 17 (53.12%), and gtfC 19 (53.37%). 120 traditional food samples (milk, yogurt, pickle, and salty pickle) were evaluated for isolation of lactobacillus. Three strains of *Lactobacillus fermentum* and four *Lactobacillus plantarum* with probiotic potential activity were isolated. Two types of bacteriocins from *Lactobacillus fermentum* and a single type of bacteriocin from *Lactobacillus plantarum* were extracted, and their molecular weights were 60, 58, and 70 kDa, respectively. In our previous study, two bacteriophages belonging to the Siphoviridae and Tectiviridae families were isolated. Real-time PCR results had shown that both bacteriocin and bacteriophage had a decreasing effect on the expression of gtfs genes. The different modes in our study for the effects of bacteriocin and bacteriophage showed that both of them had good potential as suitable options to fight dental plaque biofilms, and bacteriophages alone showed a stronger reducing effect.

1. Introduction

Tooth decay is one of the major problems in the world that begins with the formation of dental plaque. Following the formation of dental plaque, tooth decay is caused by turning sugars to acid by oral bacteria and lead to oral disease [1]. *Streptococcus mutans* is the main etiologic factor that initiates caries and has important virulence factors related to the pathogenesis of dental caries. GTFs are the key enzyme of *Streptococcus mutans* which convert sucrose into a sticky, extracellular polysaccharide which allows them to attach to a dental surface and form plaque. *Streptococcus mutans*

produces three separate Gtfs, which synthesize water-insoluble and soluble glucan [2]. GTFs have an effective role in absorption of other oral bacteria to form a dental biofilm. Biofilm can lead to increasing the antibiotic resistance of bacteria and the inability of host inflammatory cells to phagocytose biofilm cells [3]. Biological approaches are used to combat dental plaque biofilms. The use of microorganisms to promote human health and control of pathogenic bacteria has a very long history [4]. Probiotics are one of beneficial microorganisms which are able to produce bacteriocin. Bacteriocins are protein compounds with antimicrobial properties that prevent the growth of sensitive strains. These proteins have low molecular weight and are resistant to heat and are divided into 4 groups. Some bacteriocins are currently used commercially to inhibit the growth of pathogenic bacteria in food products such as fish, dairy, and meat products [5]. Another new approach used to fight and controlling bacterial biofilm is to use a specific bacteriophage against that bacterium. Bacteriophages are viruses that attack and kill bacteria [6]. These viruses are specific to bacteria and cannot attack eukaryotes. They are approximately 50 times smaller than bacteria (20–200 nm) and are ubiquitous. Due to the small size of bacteriophages, they have a considerable ability to penetrate biofilm layers [7]. Phages infect the target bacterial cell without affecting the normal flora and are naturally eliminated by eradicating the bacterial cell [8]. There are few reports on bacteriocin effect on genes level of *Streptococcus mutans* but there is not any study about the effect of bacteriophages, so in this study we aimed to compare the effect of extracted bacteriocin and lytic bacteriophage on the expression of biofilm associated genes in *Streptococcus mutans*.

2. Material and Methods

2.1. Isolation and Confirmation of S. mutans. Eighty-one dental plaque samples were taken by the dentist from patients with dental caries referred to the dental clinic of Tehran University of Medical Sciences. BHI broth (Merck, Germany) and Mitis-Salivarius Agar (QueLab, Canada) were used to isolate *Streptococcus mutans*. Suspected colonies to *Streptococcus mutans* for obtaining pure culture and biotype confirmation were followed by the tests such as catalase, carbohydrate fermentation, and urea hydrolysis [9]. *Streptococcus mutans* ATCC35866 was considered as positive control.

2.2. PCR Identification of Isolated S. mutans Genes. At first, DNA extraction of isolated *Streptococcus* mutans was followed by the described method by Hoshino et al. [10]. Nano drop device (Thermo) was used to measure the appropriate concentration of obtained DNA. Oligonucleotide sequences of primers and PCR amplification of virulence genes are displayed in Table 1. Amplification was carried out in a thermo cycler (peQlab). PCR products were introduced to 1% agarose gel electrophoresis.

2.3. Isolation and Molecular Confirmation of Probiotic Lactobacillus fermentum and Lactobacillus plantarum. One hundred and twenty samples of traditionally prepared milk, yogurt, pickles and salty pickles were collected (30 each of them) from January 2021 until June 2021. Isolation of lactobacillus strains was done by FDA protocol using enrichment in MRS broth and culturing on MRS agar. Appropriate biochemical tests were used for identification of the isolated strains. Probiotic potential activity of isolated lactobacilli was measured by acid and bile resistance. To verify that the phenotypic strains were identified as *Lactobacillus*, we used sequencing of 16srDNA gene of lactobacilli (27F : CTCGTTGCGGGACTTAA and 1522R : GCAGCAGTAGGGAATCTTC) by the PCR method. To perform a molecular reaction, we used Antonsson et al. working protocol with some modifications. The pure culture of *Lactobacillus* isolates was prepared on MRS agar medium and 2 to 3 colonies were dissolved in 50 μl of STE solution. The suspension was placed in ben-marie (96°C) for 10 minutes (boiling method). The suspension was then centrifuged at RPM 13000 for 5 minutes, and the supernatant was used for PCR [12].

2.4. Extraction of Bacteriocin from Probiotic Lactobacillus fermentum and Lactobacillus plantarum. In order to extraction of bacteriocin from *Lactobacillus*, these bacteria were concentrated and partially purified by ammonium sulfate precipitation. For this purpose, after culturing of *Lactobacillus fermentum* and *Lactobacillus plantarum*, neutralizing the effect of acid and isolating the microbial mass were performed and then the produced bacteriocin was precipitated and concentrated by precipitation with ammonium sulfate. Concentrated bacteriocin was dialyzed, and its activity was measured. SDS-PAGE technique and Bio Rad device (small gel) were used to evaluate the molecular weight of extracted bacteriocins [13].

2.5. Sampling, Isolation, and Purification of Bacteriophage. We used extracted lytic bacteriophage from our previous study [14], but the brief description is as follows.

Raw sewage from urban wastewater of Tehran was supplied, and after overnight, 10 ml of raw sewage was centrifuged for 10 min in 6000 g in 4°C. The supernatant was passing through a filter 0.22 μm. 1 ml of filtered liquid was added to a tube containing 5 ml of BHI broth with *Streptococcus mutans* (106 CFU/ml) and incubated for 24 h at 37°C. Tube was centrifuged again for 10 min in 6000 g in 4°C and supernatant filtered through a 0.22 μm filter. The double layer method (soft agar) was used to confirm the presence of lytic bacteriophage and the time that phage plaques were appeared in order to purify bacteriophage; plate surface was rinsed by SM buffer and transferred to sterile tube. Then, plaque was cut aseptically and added to tubes for repeating the above method until the isolation of pure and separated plaques. SM buffer with 20% glycerol was used to preserve bacteriophages. Host specificity, pH, and temperature tolerance of isolated bacteriophage were also measured. Finally, bacteriophage morphology was studied by using TEM [14].

Table 1: Oligonucleotide sequences of primers and PCR amplification program.

Target gene	Primer sequence (5′-3′)	PCR product (bp)	PCR programs	Ref
gtfD	F:_GGCACCACAACATTGGGAAGCTCAGTT-R: _GGAATGGCCGCTAAGTCAACAGGAT-	431	1 cycle: 95°C (5 min); 35 cycle: 94°C (45 s), 59°C–68°C (based on primers, 60 s), 72°C (60 s); 1 cycle: 72°C (5 min)	[10]
gtfB	F:-AGCAATGCAGCCAATCTACAAAT-R:_ ACGAACTTTGCCGTTATTGTCA-	96		[11]
gtfC	F:_ CTCAACCAACCGCCACTGTT-R: _GGTTTAACGTCAAAATTAGCTGTATTAG-	91		[11]

Table 2: cDNA synthesis kit manufacture.

Component	Amount
Extracted RNA	10 μl
Hexamer primer	1 μl
DEPC water	2/5 μl
5x standard buffer	4 μl
dNTPs	1 μl
RiboLock RNAase	0/5 μl
M-MLV	1 μl

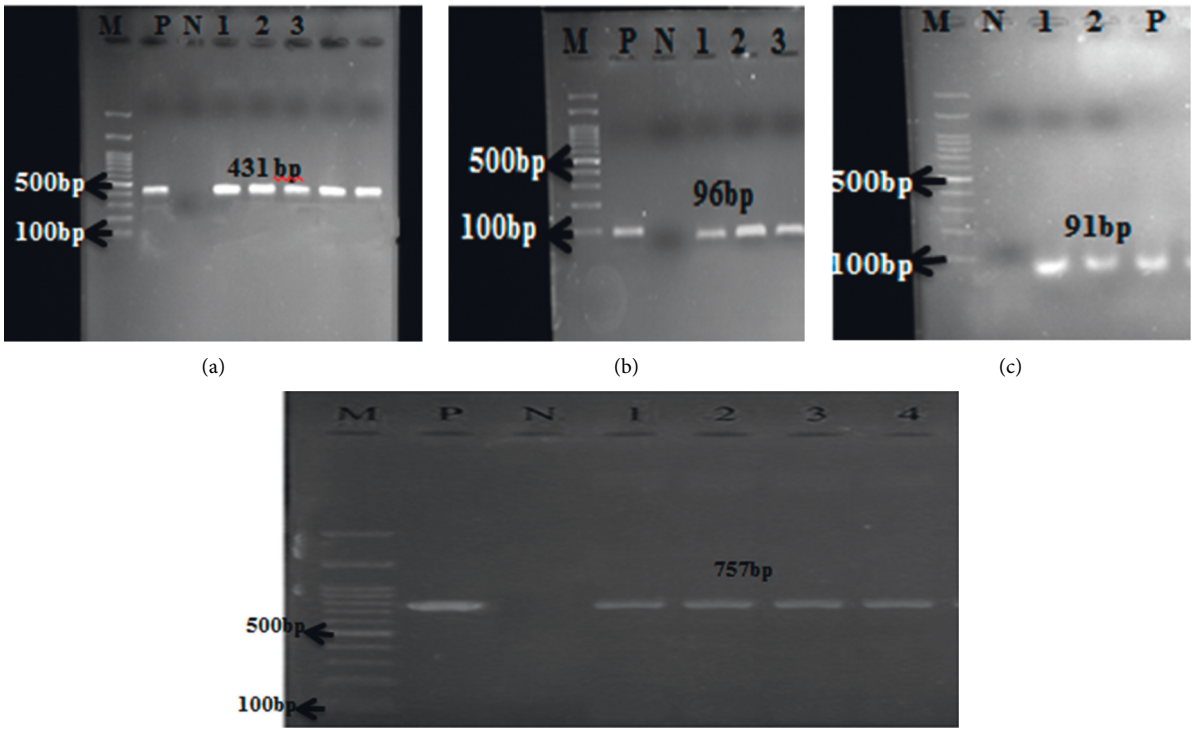

Figure 1: Results of gel electrophoresis related to PCR amplification of various genes in Streptococcus mutans strains, (a) gtfD (431 bp), (b) gtfB (96 bp), (c) gtfC (91 bp), and (d) 16srDNA gene of Lactobacillus (757 bp), M : Marker 100 bp, (P) Positive Control for Streptococcus mutans ATCC35866, and Positive Control for Lactobacillus brevis ATCC 436. (N) Negative Control (D.W, gene primers, Mastermix), Numbers: The samples contained the gene.

2.6. Real-Time Quantitative RT-PCR. The preparation of biofilm cells on microtiter plates with and without bacteriocin and bacteriophage (in 3 replicates) for RNA extraction was done according to RNA extraction process with high pure RNA isolation kit of Roche (Germany). By the cDNA synthesis kit (Thermo Scientific Revert Aid), the extracted RNA was converted to cDNA. The real-time PCR reactions (2 μl cDNA, 1 μl both forward and reverse primers, 7.5 μl SYBR green PCR master mix, and 5.5 μl DEPC water) were performed on the Rotor-Gene *Q* (Qiagene-6000) instrument

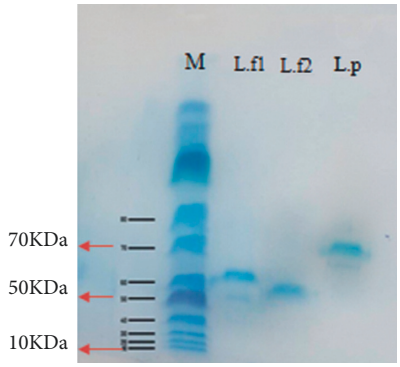

Figure 2: Bacteriocins isolated from two strains of *Lactobacillus fermentum* (L.f1 and L.f2) with a molecular weight of 60 and 58 kd, respectively, and bacteriocins isolated from (L.p) *Lactobacillus plantarum* with a molecular weight of 70 kd using SDS-PAGE electrophoresis.

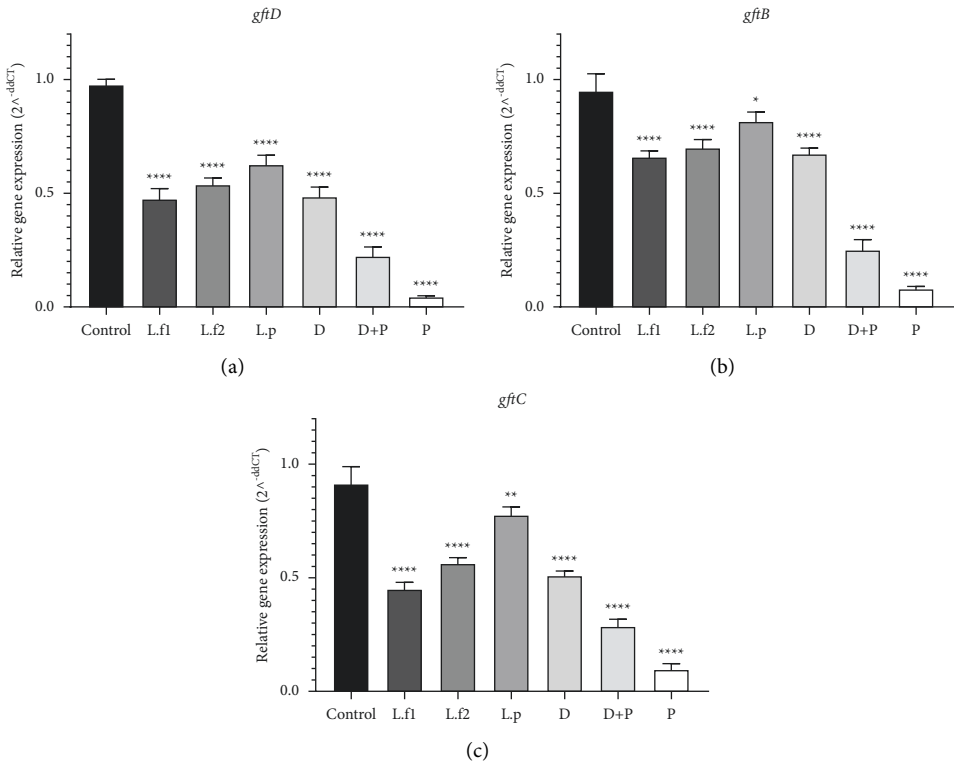

Figure 3: Evaluation of the effect of bacteriocins isolated from *Lactobacillus fermentum* 1 (L.f1), *Lactobacillus fermentum* 2 (L.f2), and *Lactobacillus plantarum* (Lp), the sum of all three bacteriocins isolated (D), the sum of all three bacteriocins isolated by lytic bacteriophage (P Specific ($D+P$), and specific lithic bacteriophage (P) on the expression of native gtf gene. (a) gtfD, (b) gtfB, and (c) gtfC) of isolated *Streptococcus mutans* from decayed dental plaque. Comparison of gene expression level with control mode shows a significant reduction level.

and SYBR Green PCR Master Mix (Qiagene). 16srDNA gene was used as a housekeeping gene.

3. Results

For isolation of *Streptococcus mutans*, out of 81 dental plaque samples, 32 (39.5%) had positive results. Molecular analysis of the gtfD, gtfB, and gtfC genes in *Streptococcus mutans* showed that all isolates had the gtfD gene. The frequency of the studied genes was as follows: gtfB17 (53/12%) and gtfC19 (53/37%) (Figures 1(a)–1(c)). Out of 120 samples of milk, yogurt, pickle, and salty pickle from different region in Tehran, 153 isolates of *Lactobacillus* were obtained and confirmed by sequencing of 16srDNA (Figure 1(d)) gene. 18 (11/76%) strains including 4(22/22%) *Lactobacillus plantarum* and 3 (16/66%) *Lactobacillus fermentum* had probiotic potential activity. The most isolated *Lactobacilli* were obtained from milk 67 (43/79%).

The results of extraction and partial purification of bacteriocins for probiotic strains of *Lactobacillus fermentum* and *Lactobacillus plantarum* are shown in Figure 2. Two probiotic isolates of *Lactobacillus fermentum* and one isolate of *Lactobacillus plantarum* had the potential to produce bacteriocin. Bacteriocins isolated from *Lactobacillus fermentum* had a molecular weight of 60 and 58 kDa, respectively, and bacteriocins isolated from *Lactobacillus plantarum* had a 70 kDa molecular weight.

Real-time results showed that in the presence of bacteriophages, the expression of used genes was highly reduced. Graph pad prism9 software was used for statistical analysis of data. Due to the normality of the data, ANOVA-one way test was chosen to compare the means ($p < 0.0001$) (Figure 3).

4. Conclusion

The oral environment is the shelter of a large and diverse number of bacterial microorganisms that are considered as flora. There are more than 700 bacterial species known in the oral cavity, of which 40 species belonged to cause caries. *Streptococcus mutans* is considered as one of the most important etiological factor for tooth decay [15]. In this study, 32 (39.5%) *Streptococcus mutans* was isolated. There was a significant relationship between caries and *Streptococcus mutans* ($p < 0.05$). Various studies have been performed on the relationship between *Streptococcus mutans* and tooth decay index, especially in the preschool children in Iran and abroad. The study results of Neves et al. [16], Bezerra et al. [17], Babaeekhu et al. [18], and Bashirian et al. [19] indicated that *Streptococcus mutans* is the main pathogen to dental caries and frequency of this bacteria is more than other oral Streptococci in many cases, especially in children under 7 years old. In this study, all isolates contained *S. mutans*-specific gtfD (Figure 1(a)) because it was specific for *Streptococcus* mutans. Gharakhani in 2019 by studying 61 dental plaque samples of people with different ages reported 19 isolates (41.7%) with gtfB gene and 8 (4.1%) with gtfC gene [20]. However, the relationship between gtfS genes expression and the quantity of ECP produced differed among clinical isolated of *Streptococcus mutans*. In a normal situation, the existence of various enzymes can influence expression and activity of genes involved in biofilm formation of *Streptococcus mutans*.

Lactobacilli are a heterozygous group of lactic acid bacteria and are especially important because of their importance for improving health, such as preventing colonization of the intestines by pathogenic bacteria and producing metabolite which are able to inhibit the growth of pathogenic bacteria [21]. Lactobacilli are also found in a variety of fermented foods. Bacteriocin is one of the major metabolite of LAB bacteria that it is commercially produced and used in the food industry as a food preservative [22]. To investigate the effects of bacteriocin on the expression level of gtf genes, at first we isolated *Lactobacillus fermentum* and *Lactobacillus plantarum* from 120 traditional foods (milk, yogurt, pickle, and salty pickle) which are usually as common consumed food by the people.

Of the 120 samples, 153 isolates of *Lactobacillus* were obtained, of which three isolates were *Lactobacillus fermentum* and four isolates were detected as *Lactobacillus plantarum* by probiotic potential activity.

SoltanDallal from pickle and salty pickle in Iran [23], Yu in China from traditional Sichuan province pickles [24], Nguyen from traditional fermentation vegetables in Vietnam [25], Parsaeimehr from traditional yogurt in Iran [26], and Yeshambel from cow milk and milk products [27], isolated a large number of probiotic bacteria that most of them belonged to LAB. Due to differences in culture, geography, and vegetation, the frequency of probiotic strains is definitely different but all data refer to beneficial effect of them to inhibit and combat by pathogenic bacteria. In the current study, we isolated two bacteriocins from *Lactobacillus fermentum* which had molecular weight of 60 and 58 kDa, respectively, and bacteriocin isolated from *Lactobacillus plantarum* had a molecular weight of 70 kDa. Adebayo in 2013 extracted bacteriocin with a molecular weight of 70 kDa from *Lactobacillus fermentum* by ammonium sulfate precipitation and dialysis [28]. Sing in 2013 observed 78 kDa extracted bacteriocin from *Lactobacillus fermentum* by the SDS-PAGE method. *Lactobacillus plantarum* in Chun study had 3.5 kDa and 4.7 kDa molecular weight by Tris-Tricine SDS-PAGE methods [29]. Due to the differences in the native of the strains and the differences in the ability to produce bacteriocins, the difference between the molecular weights of the bacteriocins certainly seems to be normal.

Phage therapy is considered a viable alternative for the treatment and control of pathogenic bacteria [30]. In this study, we tested the ability of two extracted phages from our previous study (extracted bacteriophages belonged to the Siphoviridae and the Tectiviridae family of viruses) [14] to reduce expression of the gene involved in biofilm for the first time. Also, we made an effort to investigate the effect of isolated bacteriocin and bacteriophage on gtfD, gtfB, and gtfC genes expression level simultaneously. Our results showed that lytic bacteriophages in comparison with bacteriocins had a strong inhibitory effect on reducing the expression of genes used in this study. The total effect of bacteriocin and bacteriophage together, in comparison with the effect of bacteriocin alone, was in the next order of reduction effect. Among of isolated bacteriocin, the bacteriocin extracted from *Lactobacillus fermentum* 1 had more reduction effect in comparison with two other bacteriocins. Before Tahmourespour in 2011 [31], Raj in 2017 [32], Ahmed in 2014 [15], and Wasfi in 2017 [33] have shown the decreasing effect of isolated probiotic Lactobacilli such as *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Lactobacillus Casei* on gtfs genes in *Streptococcus mutans* which is in accordance with the results obtained from our current study and showed that bacteriocins as small antimicrobial peptides are one of the most important components of probiotics in the fight against pathogens, especially *Streptococcus mutans*. However, there is no another study about the effect of bacteriophage on reducing the expression of genes associated in *Streptococcus mutans* biofilm but studies on the isolation of *Streptococcus*

mutans lytic bacteriophages and its inhibitory effect on its biofilm formation are very limited, and the results are mentioned in the discussion section of our previous study. However, the results showed a very effective role of bacteriophage on growth inhibition and inhibition of *Streptococcus mutans* biofilm.

Despite many advances in the health and dental industry, tooth decay is still a main problem. Results of our study and other previous studies showed that traditional foods can be considered as good sources of probiotic organisms. Proper selection of probiotic bacterial strains can be one of the most effective selection methods to combat biofilm and reduce the formation of dental plaque. Phage therapy is another way to solve the problem of antibiotic resistance and fight biofilm formation to help treat and kill the desired bacteria and is more effective to decrease the biofilm compared with antimicrobial therapy with synthetic antibiotics. And according to the obtained results, they can be viewed in the future as components in the oral health industry.

Data Availability

The data used to support this study are included within the published paper.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Zahra Rajabi and Rouha Kermanshahi were responsible for study concept and designing. Zahra Rajabi, Rouha Kermanshahi, and Mohammad Mehdi Soltan Dallal were responsible for data acquisition. Mohammad Reza Afradi and Donya Alinejad prepared the sample. Zahra Rajabi, Yousef Erfani, and Reza Ranjbar analyzed and interpreted the data. Zahra Rajabi and Reza Ranjbar drafted the manuscript. Finally, the paper was revised by Reza Ranjbar.

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Research Article

Effect of Apple (*Malus domestica*) Stem Cells on UVB-Induced Damage Skin with Anti-Inflammatory Properties: An *In Vivo* Study

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Objective. Apple (*Malus domesticus*) stem cells have beneficial effects in preventing photodamages caused by UVB responsible for inflammation in different stages of various skin disorders. This study aimed to investigate Apple stem cells' anti-inflammatory and repairing effects on UVB-induced rat dorsal skin damage via microscopic and macroscopic analyses. *Materials and Methods*. Therapeutic effects of the apple stem cells (ASCs) extract were evaluated after UVB irradiation with macroscopic and microscopic studies, including pathological analysis, inflammatory cytokines measuring, and biometric studies containing investigation of thickness, density, erythema, melanin, sebum, and moisture content of epidermis and dermis layers in rat models. After biometric studies, skin samples were taken for histopathologic and biochemical analyses. *Results*. ASC extract could attenuate infiltration of inflammatory cells caused by UVB and ameliorate collagen regulation of the photodamaged skin. In addition, improved skin biometrics was considerable, such as reducing thickened epidermal and dermal layers compared to other rat groups. Furthermore, moisture content enhancement of the skin showed clinical advantages in treating damages and inflammation. Furthermore, TNF-α expression was downregulated after ASC application. ASC extract could treat UVB damages and indicate anti-inflammatory effects in animal models. *Conclusion*. The ASCs can be an appropriate candidate for treating inflammation and damages induced by UVB in clinical studies.

1. Introduction

About twenty-five percent of people suffer from different kinds of skin diseases. Inflammatory skin diseases are common forms of skin diseases, for example, atopic dermatitis, psoriasis, and allergic contact dermatitis [1]. One of the adaptive reactions is inflammation, which involves soluble mediators. Multiple defense cell types respond to harmful stimuli by removing infectious agents and regulating tissue homeostasis. These active defense cells release and produce inflammatory mediators, including interleukin IL-1β and TNF-α [2]. Also, one of the contributing factors to photodamage and skin carcinogenesis is exposure to ultraviolet (UV) radiation. Dysregulated inflammation has a vital role as a crucial mechanism in the destructive effects of UV irradiation [3]. These damages are known as photodamage and photoaging related to solar irradiation. Some damages characterize brown spots, irregular pigmentation, dryness, elasticity reduction, wrinkling, leathery look, and the development of coarse solar scars [4, 5]. The UV radiation, particularly UVB, has increased skin damage with long-term consequences such as skin inflammation,

photodamage, and photoaging, which are severe concerns for the health of humans due to the depletion of stratospheric ozone, which is the critical reason for incidents on the earth [6]. Acute inflammatory skin problems such as cellular apoptosis and erythema are also caused by UVB. This radiation can also cause the promotion of UVB-induced proinflammatory enzymes, and following activation of a related signaling pathway (COX-2), it produces specific inflammatory mediators containing various cytokines and prostaglandins (PGs). Cascades of COX-2 are reasons for pain, growth of cells, and progression of tumors.

Similarly, COX-2 cascades mediate the inflammatory process. It has elucidated the importance of inflammation in the pathogenesis of skin disorders caused by UVB exposure [7]. Today, the used drugs to treat skin inflammation are topical and systemic nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. Despite this, the long-term duration of inflammatory disease treated with these agents leads to adverse reactions, including maceration, folliculitis, pruritus, irritations, dryness, military, hypertrichosis, acneiform eruptions, and allergic reactions contact dermatitis, stretch marks, and hypopigmentation. The side effects of current drugs cause to make an appropriate opportunity due to more minor toxic effects for numerous medicinal plants, which have an abundant biological function. The knowledge of using plants and plant extracts in disease prevention and treatment has been developed through continuous contact of humans with their natural environment since ancient times [2, 8]. Plants are cultivated on a large scale which is a systematic method for producing plant-derived compounds. However, high-priced harvesting and extraction methods make the procedure often economically unsustainable. In spite of this, most plants with valuable compounds have not been domesticated. Also, the wild population of these effective plants is limited. The low levels of target molecules and plant sluggish growth decline production potentiality [9].

Apple (*Malus* sp., Rosaceae) stem cells consist of many polyphenols, quercetin glycosides (flavonols), hydroxycinnamic acids, and oligomeric procyanidins, dihydrochalcones, and catechins, also anthocyanins in red apples and triterpenoids in peels of apples [10–12]. Several results proposed that apples have a broad spectrum of activities related to biological features. Production of apple compounds could be effective in respiratory problems (i.e., asthma), cancer prevention, diabetes mellitus, and cardiovascular disease. The compounds mentioned above contain antioxidants activity, anti-inflammatory mechanisms, carcinogenesis, antimutagenicity, antiproliferative pathways, changes of signal transduction activity, apoptosis-inducing, and new mechanisms on innate immunity [13]. The obtained active ingredients from apple (*Malus domesticus*) cells suspension cultures were included in the anti-aging ingredients. These plant stem cells regenerate the skin, are resistant to aging, and affect the viability and apoptosis of human stem cells, but the mechanism is unknown [9, 14]. In this study, the promising therapeutic effects of topical application of *Malus domesticus* were investigated on biophysical and biometrics skin analyses and histopathological studies in the dorsal skin of animal models exposed to acute UVB irradiation, which has been explored for the first time to evaluate the effect of apple stem cells extract on UVB-induced injury.

2. Materials and Methods

2.1. Materials. The current study purchased extracts of apple (*Malus domestica*) stem cells from Mibelle Biochemistry. Xylazine (Alfasan, Holland, 2%), ketamine (Bremer Pharma GmbH, Germany, 10%), and the rat tumor necrosis factor-α ELISA kit were purchased from ZellBio GmbH (Germany), and betamethasone topical cream 0.1% was purchased from Darou Pakhsh (Iran). Tewameter (TM300, Courage, and Khazaka, Germany), corneometer (CM825, Courage, and Khazaka, Köln, Germany), mexameter (MX18, Courage, and Khazaka, Köln, Germany), sebumeter (SM815, Courage and Khazaka, Köln, Germany), skin ultrasound imaging system (Skinscanner-DUB, taberna pro medium GmbH, Germany), and cutometer (MPA 580, Courage & Khazaka, Germany) are used for skin parameters analysis.

2.2. Animals. Twenty-four adult male rats (*Wistar*, weighing 275 ± 25 g) were obtained from Tehran University of Medical Sciences Laboratory Animal Center and were kept at the Animal Center. Animals were accustomed for two weeks and kept in polypropylene boxes with wood-lined bedding, which was changed daily, with 12 hours' light/dark cycle, and controlled temperature (22–24°C). Food and water were given *ad libitum* in experiment duration. In vivo studies were performed in compliance with National Institutes of Health guidelines for the welfare of experimental animals. The Ethics Committee approved the protocol of the Faculty of Pharmaceutical Science of Tehran University of Medical Science (IR.TUMS.VCR.REC.1399.455). The hair on the dorsal areas of the rats ($4 \times 4\,\text{cm}^2$) was removed by using a depilatory cream then. Irradiations started after 3 days. Finally, the animals were euthanized, and dorsal skin samples were taken for histopathological and biomarkers studies.

2.3. Experimental Design. The rats were randomly assigned into four groups (Sham, Control (-/UVB), betamethasone (betamethasone/UVB), and ASCs (ASCs/UVB)) ($N = 6$). The sham group animals were not exposed to UVB without any topical treatment. A pilot study was conducted to distinguish possible allergic reactions using two non-irradiated animals treated with ASCs extract. Sham groups are analyzed histopathologically and macroscopically, and TNF-α and TGF-β1 are assessed. The animals of other groups were exposed to UVB irradiation ($180\,\text{mJ/cm}^2$) applied once daily for one hour in 7 consecutive days. After UVB-induced damage, rats were treated for their group one daily for 7 days. As mentioned above, the control group was treated without any formulation. The betamethasone group was treated with Betamethasone 0.1% cream, and rats in the ASCs group were treated with the extract of apple stem cells. Biophysical and biometrics skin values were analyzed on days 0 (exactly before any topical treatments), 1, and 7. Moreover,

immunohistological investigation and histopathological studies were evaluated on days 1 and 7 in control, betamethasone, and ASC groups.

2.4. Irradiation. UVB radiation was conducted as mentioned above. The source of UVB irradiation (Philips TL20W/01 RS lamp, Holland) was placed 20 cm above rats, which emitted the peak wavelengths of 313 nm narrowband UVB [15, 16]. 80% production of total UV radiation was measured through the radiometer sensor (UV: SED005, and UVB: SED240). The irradiation rate of UVB was 0.18 mW/cm^2. Also, the dose of UVB utilized in the experiment was 0.3 J/cm^2. Before being exposed to UVB irradiation, rats were anesthetized with an intraperitoneal injection (Ketamine/Xylazine).

2.5. Administration. The ASC extract (20 mg with a concentration of 20 mg/ml) was topically used on the skin of rats once daily for 7 days in the ASC group. Moreover, betamethasone cream (500 μg with a concentration of 1 mg/g) was administered on the dorsal areas of rats in the betamethasone group once daily for 7 days. Pilot studies of our research group and previous studies showed that the amount of topical administration was following dose-response [17, 18]. All of the treatments were started after 7 days of UVB irradiation.

2.6. Histologic Analysis. The skin samples were fixed in buffered formaldehyde solution (10%), dehydrated in ethanol solutions, diaphenized in xylene, embedded in paraffin, and then cut into 5 μm sections stained with hematoxylin & eosin for a histological evaluation. The histopathological changes such as damages of different layers of skin also epidermis thickness were assessed randomly selected histological fields (optical microscopy, Olympus BX51, Tokyo, Japan) [19].

2.7. Tumor Necrosis Factor-α in the Cutaneous Tissue. Before the dorsal skin was surgically collected, animals were euthanized. Also, samples homogenated in 500 μL of phosphate-buffered saline (1.5 mM KH_2, 3 mM KCl, 10 mM Na_2HPO_4, and 137 mM NaCl, pH 7.4). Then, skin specimens were centrifuged (10.000 g, 4°C, 10 min). The produced supernatants were utilized for quantification of TNF-α with the ELISA Kit (ZellBio, GmbH, Germany).

2.8. Real-Time PCR Analysis. Total RNA Kit (RNA simple Total RNA Kit, CHN) divides total skin RNA, quantified by measuring the absorbance at 260 nm. Then, the cDNA Synthesis Kit (VILO™ cDNA Synthesis Kit, Thermo, USA) was used to reverse transcription RNA. Primers of TGF-β1 (forward primer (5′ to 3′) CGCCTGCAGAGATTCAAGTC and reverse primer (5′ to 3′) GCCCTGTATTCCGTCT CCTT) were designed using the sequence detection system. Despite accomplishing the quantitative expression of mRNA, real-time PCR elaboration was carried out on a sequence detection system (ABI Prism 7500, USA) with a Fast Start Universal SYBR Green Master (Rox, Roche, Switzerland) for amplification reaction assays at the optimal concentration (40 times cycles, 15 s at 95°C, 60 s at 60°C, and 2 min at 72°C). Data were analyzed and standardized by thermal cycler software, and each sample was tested three times.

2.9. Biometric Analysis of the Skin. Rats were anesthetized with an intraperitoneal injection (ketamine/xylazine) on days 0, 1, and 7 after induced damages by UVB irradiation. Afterward, the skin parameters of rats containing transepidermal water loss (TEWL), hydration of stratum corneum, melanin, erythema, sebum, thickness, density, and elasticity were assessed through particular apparatus such as the tewameter, corneometer, mexameter, sebumeter, and skin ultrasound system. Investigations are analyzed after collecting the results.

2.10. Statistical Analysis. Data were analyzed using analysis of variance, followed by Duncan's multiple range test for comparison between mean values at a significance level of $p < 0.05$ (SPSS Statistics 20 software, SPSS Inc., USA).

3. Results

3.1. Macroscopic Evaluation. Macroscopic evaluations of the UVB effect on rat dorsal skin are shown in Figure 1. The dorsal skin appeared healthy in the sham group without abnormality or erythema. The dorsal skin was irradiated for 7 days with UVB irradiation in other groups. The induced photodamage features were sunburns, deep-wrinkled, dark-brown color, and leathery skin (with flesh-colored lesions). The appearance of the skin was relatively improved after the first day of treatment with ASCs and betamethasone, but the signs of UVB remained.

Additionally, the undesirable skin appearance of UVB-induced was significantly improved in treated groups with apple stem cells and betamethasone. The skin was significantly softer in the ASCs group, and no signs of sunburns or erythematous changes were detected. The observation in the ASCs group was nearly close to the sham group on day 7. Moreover, in the betamethasone group, the improved dorsal skin was similar to treatment with ASCs (Figure 1).

3.2. Effect of Apple Stem Cells in Comparison with Betamethasone Cream on the UVB-Induced Skin Damage

3.2.1. Measurement of Transepidermal Water Loss (TEWL) on the UVB-Induced Skin Damage. TEWL was evaluated (Tewameter®, TM300, Courage, and Khazaka, Germany) to detect the baseline TEWL value (about 9 ± 2 g/h/m^2) and the skin barrier function [20]. TEWL was determined about $500 \pm 50\%$ after 7 days of the UVB irradiation. The extent of TEWL was gradually decreased in each group. On the other hand, TEWL levels had no significant difference in the ASCs group compared to the betamethasone and control groups on day 7. However, the TEWL level in the ASC group was decreased more than in other groups ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$) (Figure 2(a)).

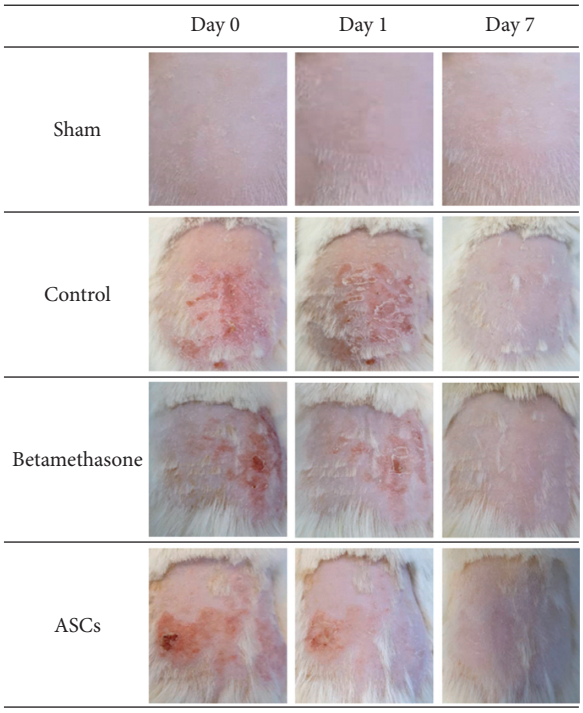

Figure 1: Macroscopic changes of rat skin in sham, control, betamethasone, and ASC groups on days 0, 1, and 7.

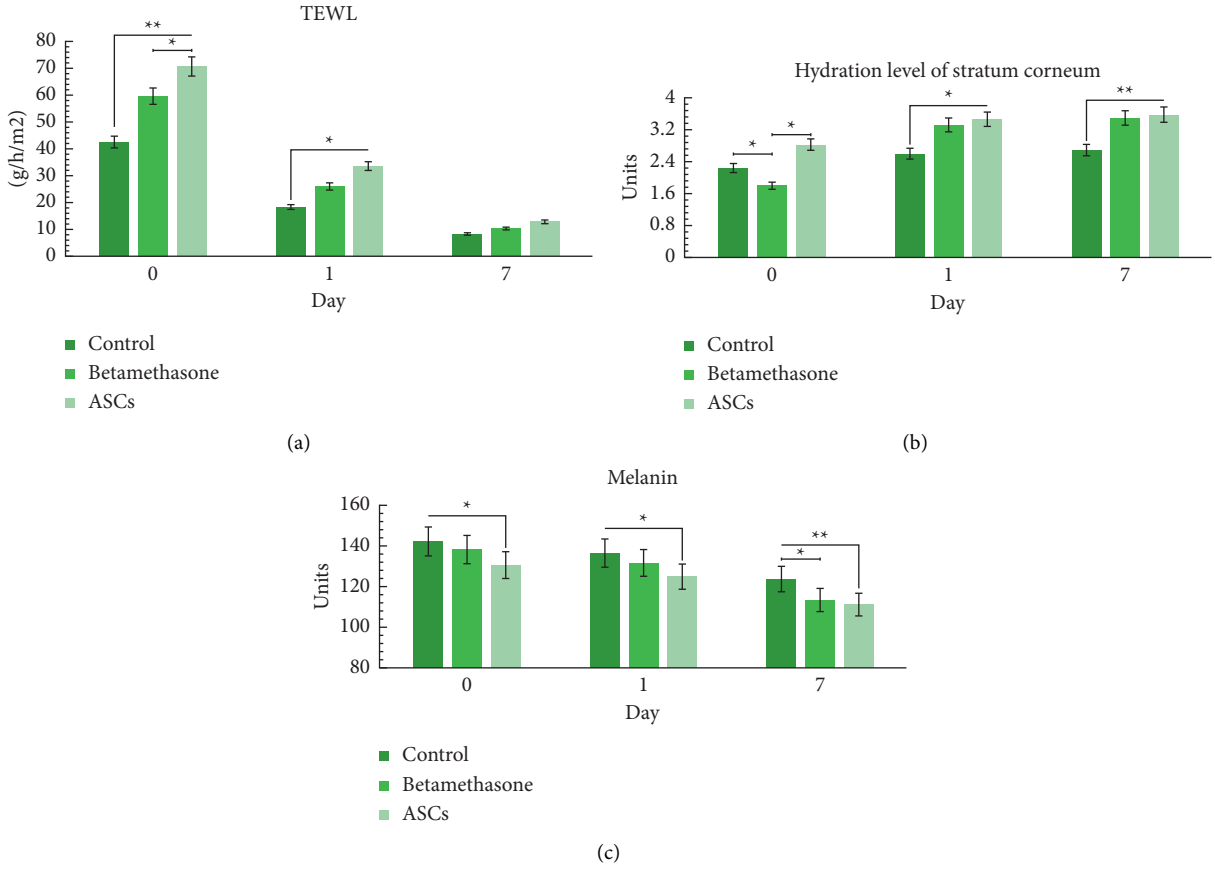

Figure 2: (a) TEWL level as an indicator of water loss, (b) hydration level in stratum corneum, (c) melanin content of UVB-irradiated skin in treated (betamethasone and ASC groups) and control groups on day 0, 1, and 7. Error bar: mean ± SD ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$).

3.2.2. Measurement of Skin Moisture Content on the UVB-Induced Skin Damage. Evaluation of moisture contents (Corneometer®, CM825, Courage, and Khazaka, Köln, Germany): in rats irradiated with UVB for 7 days (an hour per day), the moisture content of rat skin was steadily reduced in the betamethasone and control groups from day 0 to 7. Also, the topical application of ASCs extract slightly ameliorated the moisture loss of photodamaged skin, although TEWL was decreased in all treated groups ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$) (Figure 2(b)).

3.2.3. Measurement of Melanin on the UVB-Induced Skin Damage. Melanin values were measured (Mexameter®, MX18, Courage, and Khazaka, Köln, Germany) from days 0 to 7 in the treatment duration of treated groups. In 7 days duration of the experiment, melanin was decreased value in ASCs, betamethasone, and control groups.

The values attributed to melanin amount were declined in betamethasone and ASCs groups compared to the control group. The amount of the melanin was 113.2 ± 0.1 in the case of the betamethasone group, which was close to the ASC group (111.1 ± 0.1), but both values were lower than the control group with 124 ± 0.2 ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$) (Figure 2(c)).

3.2.4. Measurement of Erythema on the UVB-Induced Skin Damage. The measurement confirmed the observation of changes in the degree of erythema. Skin erythema was increased initially due to the UVB exposure in 7 days and had gradually reduced to a normal condition. Although the difference was not significant between groups in erythema with entire treatment duration in the control and ASCs groups, the values of the arbitrary units indicated the severity of erythema were lower in control and ASC groups than the betamethasone group ($^{*}p \leq 0.05$) (Figure 3(a)).

3.2.5. Measurement of Sebum Contents on the UVB-Induced Skin Damage. Skin sebum content was measured by a sebumeter® (SM815, Courage and Khazaka, Köln, Germany), which was substantially decreased after UVB exposure in 7 days. The betamethasone treatments slightly increased sebum content by about 30% without any significance. The sebum level dramatically increased after 7 days in the ASCs group, and also, 150% rise-up in sebum content was observed from a value of 3.8 to 9.7 μg/cm^2. A 130% increase of sebum could be detected in the control group in 7 days. It is noticeable that sebum amelioration was found in all treated groups but, the most significant induction showed in the ASC group ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$) (Figure 3(b)).

3.2.6. Measurement of Elasticity on the UVB-Induced Skin Damage. The standard deviations and means of the R parameters were obtained in three groups. Statistically, the elasticity differences were found between the control, Betamethasone, ASCs groups for every R parameter (R7: ratio of elastic recovery to the all deformation, R5: net elasticity, R2: overall elasticity of the skin). In particular, the R7 parameter was the most critical parameter of elasticity for measuring (Cutometer, MPA 580, Courage & Khazaka, Germany) skin aging. In the control and betamethasone groups, differences between changes of R parameters were not significant from day 0 to 7. On the other hand, the number of R parameters, particularly R7, was consistently increased in the ASC group compared to other groups ($p \leq 0.05$) (Table 1).

3.2.7. ASC Effect on the Decrement of TNF-α Activity in UVB-Induced damage on the Skin. UVB-irradiation significantly elevated the concentrations of cytokines such as TNF-α in the skin. After UVB-irradiation, ASC extract treatment indicated anti-inflammatory effects in topical administration such as inhibition of TNF-α expression. Also, the ASC group showed an anti-inflammatory effect on day 1, and the concentration of TNF-α was similar to day 7. However, it had a meaningful difference with the sham group. The TNF-α concentration in the skin was upregulated moderately in the control group. On the contrary, the expression of TNF-α in the betamethasone group was steadily decreased, but TNF-α expression was decreased significantly in the ASCs group in 1 day ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$) (Figure 4(a)).

3.2.8. Measurement of Skin Thickness and Density on the UVB-Induced Skin Damage. The skin thickness and density were measured by a skin ultrasound imaging system (Skinscanner-DUB, taberna pro medium GmbH, Germany). Total thickness and density of dorsal skin of rats were increased in all groups after 7 days of UVB exposure. The thickness and density of epidermis and dermis layers were measured on days 0, 1, and 7. The thickness and density were gradually decreased in all treated groups. Nevertheless, the thickness of the epidermal and dermal layer in the ASC group was thinner than the dorsal skin layers in the control and betamethasone groups. Also, the density of epidermis and dermis of the control and betamethasone groups were relatively declined, which was lower than the density of dorsal skin in the ASC group. To summarize, the results demonstrated that the density and thickness were decreased in all treated groups, but the reduction in the ASC group was more significant (Figure 4(b)). Thickness and density changes were evaluated in the treated group (Tables 2 and 3).

3.3. Pathological Analysis. Pathological analysis showed that the skin layers, without any UVB exposure, were observed in the dorsal area of the sham group as normal and healthy skin without any apparent abnormal structures. Moreover, no changes were detected in the epidermis, dermis, and hypodermis layers. Similarly, the thickness of the epidermis layer was standard. Moreover, the number of inflammatory cells and polymorphonuclear leukocytes were standard in the epidermal layer. Collagen fibers were normal in skin layers without any degradation. Histopathological results demonstrated that the thickness of the dermis and epidermis

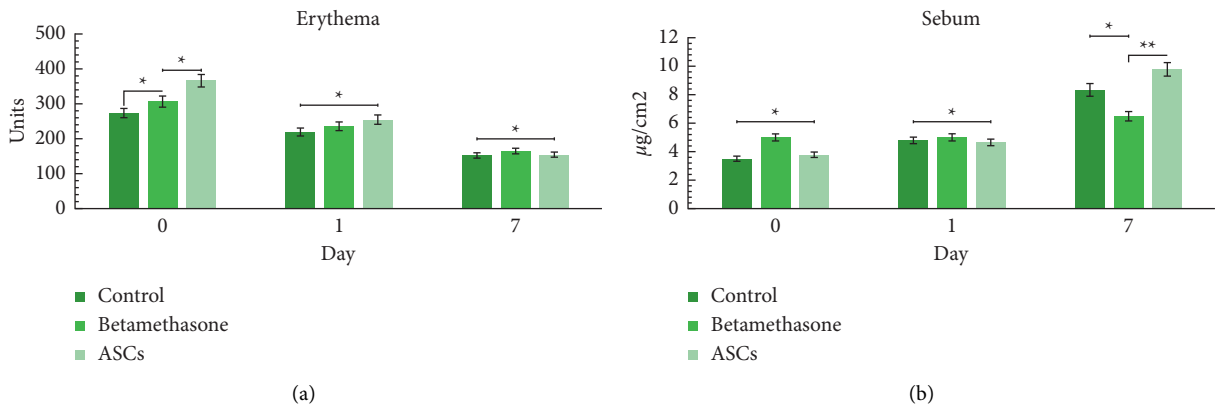

Figure 3: (a) Erythema formation and (b) sebum content of UVB-irradiated skin in treated (betamethasone and ASCs groups) and control groups on days 0, 1, and 7. Error bar: mean ± SD ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$).

Table 1: Percentage of overall elasticity (R2), net elasticity (R5), and the ratio of elastic recovery (R7) between three groups on days 0, 1, and 7 ($p \leq 0.05$).

Group	Elasticity (%)								
	Overall elasticity (R2)			Net elasticity (R5)			Ratio of elastic recovery (R7)		
	Day 0	Day 1	Day 7	Day 0	Day 1	Day 7	Day 0	Day 1	Day 7
Control	0.514	0.571	0.576	0.4	0.402	0.381	0.239	0.209	0.203
Betamethasone	0.51	0.546	0.595	0.358	0.346	0.425	0.193	0.193	0.233
ASCs	0.508	0.572	0.75	0.356	0.386	0.575	0.203	0.216	0.327

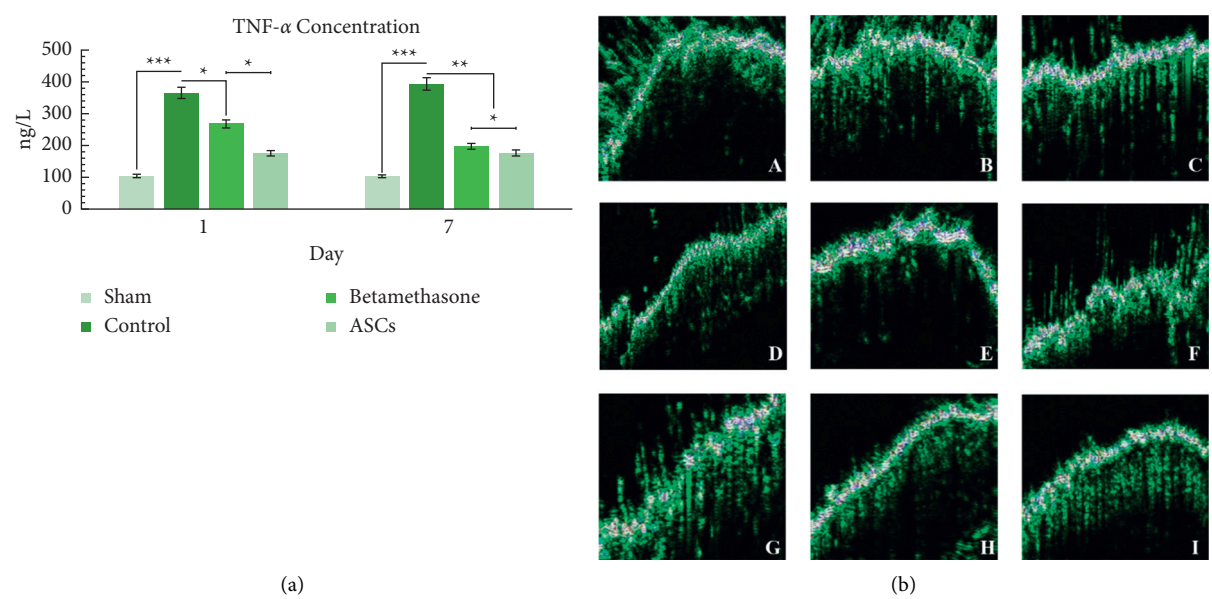

Figure 4: (a) Samples of sham, control, betamethasone, and ASC groups were collected and analyzed by TNF-α on days 1 and 7. Error bar: mean ± SD ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). (b) Thickness and density were evaluated with ultrasonography images in treated (betamethasone and ASC groups) and control groups on days 0, 1, and 7. Ultrasonography image (A; 0, B; 1, and C; day 7) in control, (D; 0, E; 1, F; day 7) in betamethasone, and (G; 0, H; 1, I; day 7) in ASCs groups.

of the dorsal skin was significantly increased on day 1 after starting the treatment period in the control group. At the same time, the number of inflammatory cells and polymorphonuclear leukocytes were elevated substantially. Also, collagen fibers were irregularly arranged in the rat's dorsal skin in the control group.

In the control group, the thickness of the epidermis on day 7 was significantly enhanced more than the control group on day 1. The thickened epidermis on day 7 is approximately two times more than the control group on day 1. Moreover, the degraded collagen by UVB irradiation was substantially detected more than on day 1. Also,

Table 2: Thickness of epidermis, dermis, and complete skin in control, betamethasone, and treatment with ASC extract on days 0, 1, and 7 ($p \leq 0.05$).

Group	Density (%)								
	Epidermal density			Dermal density			Complete density		
	Day 0	Day 1	Day 7	Day 0	Day 1	Day 7	Day 0	Day 1	Day 7
Control	41.2	40.2	40.5	7.61	6.98	5.15	13.74	12.94	10.67
Betamethasone	43.52	38.52	38.96	5.83	5.63	4.42	12.65	12.74	10.37
ASCs	43.71	41.18	40.64	6.26	5.48	5.52	12.87	12.33	11.32

Table 3: Percentage of density in epidermis, dermis, and complete skin in control, betamethasone, and treatment with ASC extract on days 0, 1, and 7 ($p \leq 0.05$).

Group	Thickness (μm)								
	Epidermal thickness			Dermal thickness			Complete thickness		
	Day 0	Day 1	Day 7	Day 0	Day 1	Day 7	Day 0	Day 1	Day 7
Control	157.3	151	133.8	878	690.1	607	1035.5	841.1	740.8
Betamethasone	164	139.6	124.1	807.75	623.2	598	971.75	762.8	722.1
ASCs	173.5	146.8	122.5	818	718.7	536.8	991.5	865.5	659.3

inflammatory cells and polymorphonuclears infiltration of the dermis increased more than the control group on day 1. The thickness of the dorsal skin dermis and epidermis, exposed to UVB for 7 days before treatment duration, was relatively like the control group in the Betamethasone group on day 1 of treatment duration. Furthermore, perivascular inflammatory cell infiltration was detected.

Additionally, collagen fibers were distorted, broken, and even abnormally accumulated in the dermis of the skin. On day 7 of treatment with betamethasone, improvement of the epidermis layer was observed compared to the control group. Moreover, the thickness of the epidermis layer was decreased considerably. On the other side, inflammatory cell infiltration was significantly dropped compared to the control group on day 7. Collagen bundles were comparatively decreased in the Betamethasone group, although the collagen fibers were arranged better than fibers in the control group on day 7 (Figures 5 and 6).

In the ASC group on day 1, epidermis thickness and width changes were not significant, and also, many collagen bundles could be detected. Similarly, irregularly arranged collagen fibers were observed. Also, infiltration of inflammatory cells in the ASC group was shown and, dermis width and thickness changes were not dramatic and closed to the seventh day of the treatment period in the control group on day 1. The thickness of the dorsal skin epidermis of the ASC group has relatively plummeted after 7 days of UVB irradiation compared to the control group on day 7 of treatment duration. Furthermore, infiltration of inflammatory cells and polymorphonuclear leukocytes decreased in the dermis. The structure and density of dermal collagen fibers were improved, although collagen bundles were observed on day 7 in the ASC group.

3.4. Levels of TGF-β1 Expression in UVB-Induced Skin. Transforming growth factor-β1 (TGF-β1) levels were measured by RT-PCR in UVB-irradiated skin and sham groups. The concentration of TGF-β1 was not changed in the sham group on days 1 and 7. Also, after UVB irradiated to betamethasone and ASC groups, TGF-β1 level was decreased after a day of ASCs, and betamethasone topical administration compared to the TGF-β1 concentration in the control group. This result indicated that TGF-β1 was reduced in the control group, while after using formulations, TGF-β1 contents were increased on day 7 in contrast to day 1 in treated groups (betamethasone and ASC groups). Also, the rise-up of TGF-β1 in the ASC group was lower than the concentration in the betamethasone group ($^{*}p \leq 0.05$) (Figure 7).

4. Discussion

The physiologic response to UV light exposure displayed acute exposure thickens the epidermis, edema, burns, hyperplasia, inflammation, and erythema. In contrast, chronic exposure to UVB irradiation can cause carcinogenesis and the aging process in the skin [7]. UVB can change abundant cell signaling pathways responsible for the skin's inflammation. Furthermore, inflammatory cytokines (i.e., TNFα and IL-6) play pivotal roles in skin inflammation [21]. Several studies have exhibited that product-derived agents from natural sources would show a protective effect on UVB-damaged skin due to their different bioactive compounds. They can also combat human diseases such as carcinogenesis, cardiovascular disease, and inflammatory diseases [22]. Scientists found that NF-κB was caused transfection in human umbilical vein endothelial cells with an NF-κB reporter-driven structure. Pretreatment with polyphenol of apple extracts (24 h) substantially decreased TNF-α-mediated expression of the reporter gene. Also, a decrease of NF-κB was recognized when pretreated MCF-7 cells with apple extract for 2 hours and induced with TNF-α for half an hour [13]. Also, another group of scientists showed that the extract of ASCs affects viability and resistance of the aging process and human stem cell apoptosis. Also, *in vivo* study on 20 women aged 37 to 64 years have used the apple stem cell extract twice a day on the crow's feet

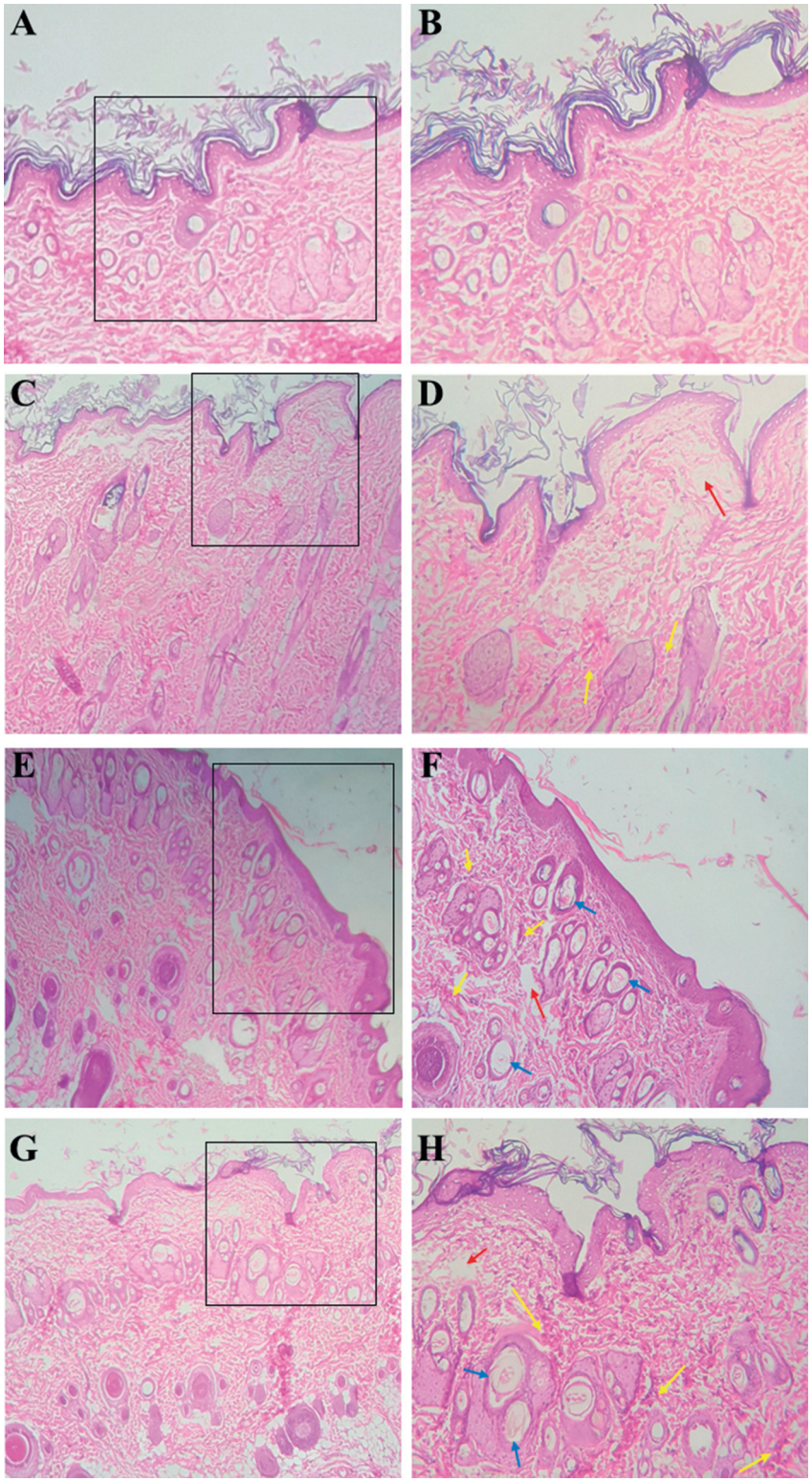

Figure 5: Histological analysis (thickness, inflammatory cell infiltration, collagen fiber regulation conditions) on day 1. Sham group (A; ×40, B; ×100), control (C; ×40, D; ×100), betamethasone (E; ×40, F; ×100), ASC groups (G; ×40, H; ×100) (red arrows: collagen necrosis; blue arrows: collagen bundles; yellow arrows: inflammatory cells infiltration).

area for 4 weeks. Clinical studies showed that 15% of wrinkle depth was reduced after the duration of administration. Furthermore, the extract was presented to be effective in human stem cells protection from UV irradiation, but the mechanism of the extract is not known [14]. Due to the promising properties of apple components in reducing inflammation, ASCs extract was used to investigate the effects of the extract on UVB skin damage. In this study, the topical

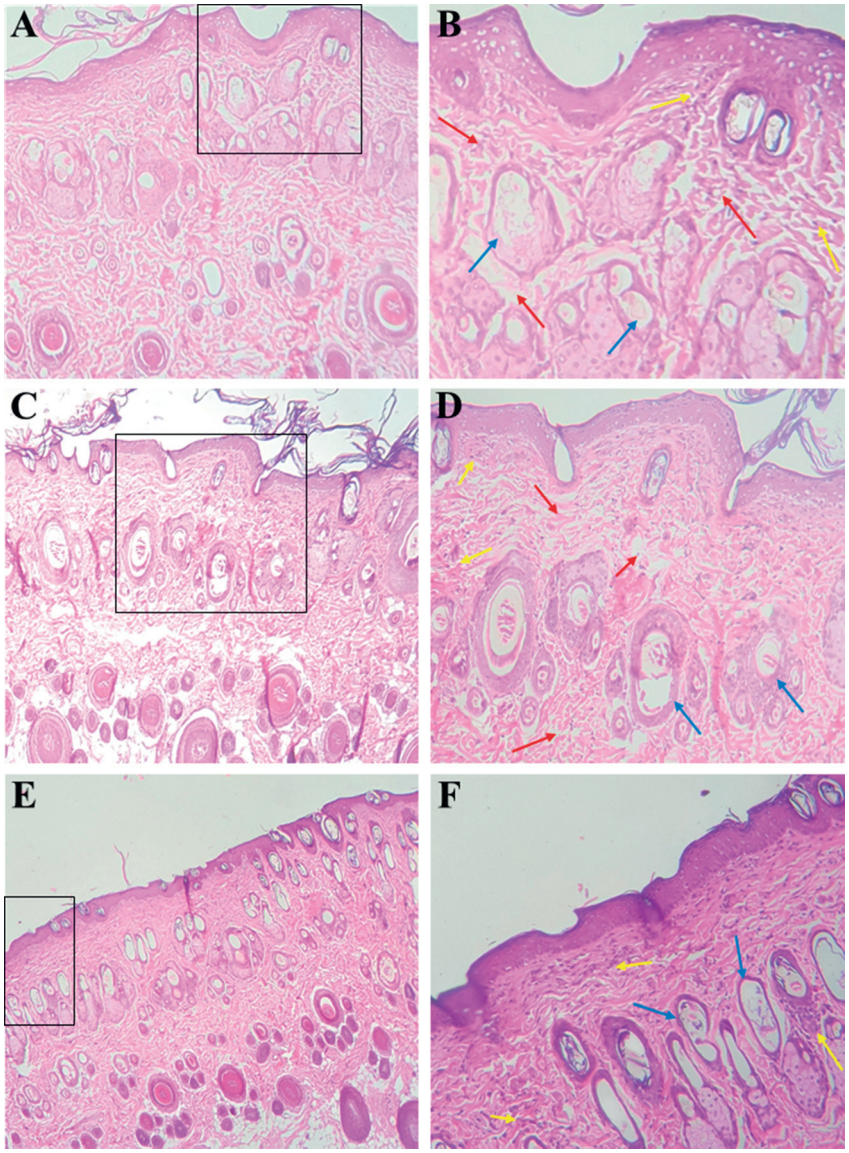

Figure 6: Histological analysis (thickness, inflammatory cells infiltration, collagen fiber regulation conditions) on day 7. Control (A; ×40, B; ×100), betamethasone (C; ×40, D; ×100), ASC groups (E; ×40, F; ×100) (red arrows: collagen necrosis; blue arrows: collagen bundles; yellow arrows: inflammatory cells infiltration).

administration of ASCs extract could ameliorate UVB-induced damages revealed by attenuation of TNF-α expression, improvement of skin structure, and return important contents of the skin. Moreover, animal studies displayed that treatment of ASCs extract could constrict skin photoaging marked by erythema, histologic changes, wrinkles, tanning, thickening, and dryness of the skin. Reich et al. demonstrated that the apoptotic dose of UVB irradiation is close to the minimal erythema dose of UVB, which means that UV-induced erythema might be an inflammatory response to the presence of sunburn and apoptotic cells [23]. The epidermis after the UVB-caused sunburn shows abnormal differentiation and proliferation, which appears to be related to the effect on skin barrier function leading to photo-aging or damage, which seems to be closely related to the effect on the skin barrier function leading to photodamage [24]. In the study of researchers Alves et al., the effect of two topical formulations of *Malus* sp. extract (containing 1.25%) and the equivalent amount of rutin (0.75%) was assessed. Also, the effect of photo-chemopreventive was evaluated on two three-dimensional (3D) skin models and then compared to 3D tissue-engineered skin models. Both formulations could protect skin against the increase of sunburn cell formation induced by UVB, including cyclobutane pyrimidine dimer formation and caspase-3 activation in both skin models. In addition, the formulations prevented the lipid peroxidation and the UVB-induced formation of metalloproteinase [24]. In the present study, a formulation based on ASC extract was prepared to ameliorate the UVB-induced damages of skin irradiated with a dose of 0.3 J/cm^2. The irradiation was continued for an hour to induce photodamages in the rat model. Also, in a study by Huang et al. (2020), the skin was irradiated with UVB a dose of 100 mJ/cm^2 for 12 minutes for evaluating smoothness and antiaging effect of facial masks

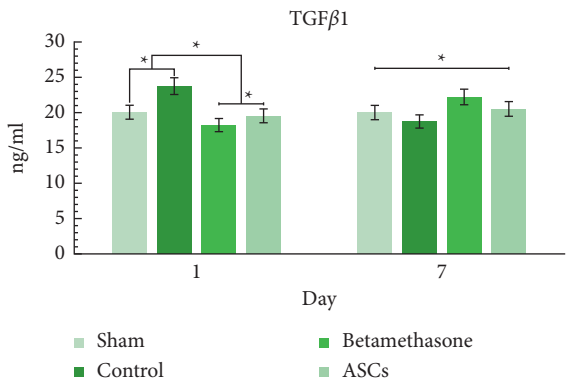

Figure 7: TGF-β1 concentration of rat skin in sham, control, betamethasone, and ASC groups on days 1 and 7. Error bar: mean ± SD ($^{*}p \leq 0.05$).

containing farnesol [6]. Mueller et al. found that apple extract including 30% phloridzin and 5% quercetin inhibited the 89% production of TNF-α in a stimulated macrophage model [25]. Moreover, Oresajo et al. demonstrated the protective role of antioxidants including vitamin C, ferulic acid, and phloretin (a potent antioxidant in apple peel extract) on human skin from the UV damages [26], Shoji et al. indicated the inhibitory effect of apple polyphenols (procyanidins and other flavonoids) was more effective than kojic acid or arbutin in melanogenesis [27]. In this study, ASCs extract was used for investigating the positive effects of apple components in UVB-induced damage and inflammation models in the abovementioned studies. The improvement of skin moisture contents was increased by administration of W/O emulsion cream based on a hydroalcoholic extract of apple seeds after 8 months due to TEWL reduction [28]. Additionally, the improvement in mechanical features of the skin was detected, for instance, roughness levels, photoaged wrinkles appearance, and smoothness. Moreover, Khan et al. used a W/O emulsion cream based on a hydroalcoholic extract of apple seeds formulation on the hyperpigmented skin in a clinical study. They found that reducing erythema and melanin amounts but increasing sebum production. In apple extract, these effects on the skin could be associated with quercetin, flavonoid, and hesperetin [29]. TEWL is also used as an indicator of skin barrier changes, consistent with the stratum corneum being the predominant obstruction for water outflow. It has been proposed that UVB disrupts stratum corneum and the rise-up of TEWL level in hairless mice [30]. Also, UVB exposure increased the dehydration and a rise-up in TEWL level of the skin attenuating the skin barrier function and reducing water content, ceramide, and hyaluronan [31, 32]. This study indicated that the ASCs extract reduced the TEWL level by ameliorating the skin function barrier. In Betamethasone and control groups, TEWL was decreased, and the skin structure reformed on day 7. However, the regulation of TEWL and skin hydration level in the ASCs group was more significant than in other groups (Figures 2(a) and 2(b)). Therefore, ASCs compounds may also improve the function of skin barriers and regulate the hydration of the skin with reduction of TEWL. Also, wrinkling may improve because of the enhancement of skin hydration and skin barrier function. Also, ASCs showed the moisturizing effects in this rat model with the regulation of skin hydration. Ultrasound images measure skin echogenicity and skin thickness in different dermis layers. Additionally, cutaneous photodamage is scored clinically. Kim et al. found the ultrasonographic differences between re-epithelialization of skin after normal skin and partial-thickness burns [33]. In addition, UVB exposure to the skin could reveal an enhancement of thickness, density, and wrinkling of the skin. Similarly, it has shown that the reduction in elasticity is crucially correlated with reductions in the level of collagen type I [34]. The present study demonstrated that the thickness of skin and density of dermal and epidermal layer was significantly increased while the elasticity was decreased after UVB exposure duration. In topical application period time, the thickness of rats was declined significantly while the elasticity was increased in the ASCs group. Therefore, amelioration of total thickness and skin elasticity were observed more significantly in the ASCs group. Moreover, erythema was caused by UV irradiation, and it could be characterized by histological (elastic fiber degeneration) and reticular hyperpigmentation. UVB-caused erythema induces wrinkle formation in mice models, which is relevant for assessing wrinkle formation. Erythema is an individual marker for UVB exposure due to the dilation of the dermal vessel and inflammation [34, 35]. In this study, erythema in the ASCs group was remarkably decreased compared to the Betamethasone group. However, the difference between ASCs and the control group was not significant in the dorsal skin of rats. Sumiyoshi et al. showed that oyster protein hydrolysates could prevent skin photodamage with anti-inflammatory effects induced by UVB irradiation by regulating the abnormal expression of MMP-1. The possible molecular mechanism underlying oyster protein hydrolysates antiphotoaging is probably associated with reducing the MAPK/NF-κB signaling pathway regulation. At the same time, the production of TGF-β is increased in the skin [34]. The accumulation of polymorphonuclear and mononuclear cells in the dermis is observed for *in vivo* response of UVB experiment in detecting vascular endothelial adhesion molecules. Cytokines cause the improvement of induced inflammation (UVB) in epidermal production. Strickland et al. studied the protein expression and IL-8 and TNF-α mRNA after UVB irradiation (24 h) in the epidermis. Moreover, the neutrophil accumulation and adhesion molecule expression were evaluated in the dermis. Finally, they found that protein and mRNA expression were formed in the non-irradiated epidermis. After UVB irradiation, protein and TNF-α (mRNA) were insignificantly elevated during 8 h and then achieved the highest level during 24 h. TNF-α was not involved in the early adhesion molecule induction due to the increased E-selectin expression before increasing TNF-α protein during 4 h but IL-8 and TNF-α may increase the inflammatory reaction [36]. The present findings displayed that exposed skin (UVB) could induce damage that initiates inflammation and changes in skin layers and stimulates inflammatory cells and polymorphonuclear leukocytes

infiltration. Also, cytokines such as TNF-α were up-regulated after UVB exposure and photo damages. However, topical application of ASCs could significantly reduce TNF-α concentration and decrease cytokine expression compared to the control group. Additionally, the sebum content was increased in the ASCs group; thus, topical administration of the formulation could improve the sebum content of the skin and reduce aging after photodamage. Hašová et al. investigated hyaluronan's human keratinocyte modulation response to UVB radiation. A single dose of UVB was irradiated to human keratinocytes (HaCaT) and instantly treated with hyaluronan for 6 and 24 hours. The UVB-mediated production of transforming growth factor β was decreased treatment by hyaluronan [37]. *In vivo* study of Xu et al. showed that the expression of collagen types I and III was increased after subcutaneous injection of 10-fold concentrated conditioned medium of dedifferentiated adipocytes, which also decrease the cell proliferation and delayed UVB-induced aging in human dermal fibroblasts. Results suggested that dedifferentiated adipocytes effects were mostly due to the secreted factors, especially TGF-β1, which has a key role in synthesis stimulation of collagen in human dermal fibroblast [38]. TGF-β1 expression in the ASCs group was increased after 7 days of treatment duration while the enhancement the level of TGF-β1 in the group that received Betamethasone, as a common treatment for inflammation, expression of TGF-β1 was significantly increased compared to control and ASCs groups. Also, topical administration with ASCs had a regulatory effect on the TGF-β1 expression that is a pivotal component in the production of elastin and photoaging processes [39, 40]. Becker et al. found that UVB irradiation could increase infiltration of the inflammatory cells in the mice paw tissue. Furthermore, cream based on Copaiba oleoresin inhibited the inflammatory cell infiltration in UVB-induced animal models [41]. Histopathological observation of Huang et al. showed that the facial masks including farnesol were administered before UVB exposure which could improve epithelialization, collagen arrangement, and collagen content [6]. Serafini et al. investigated the benefits of *Morinda citrifolia* topical application on the dorsal skin of mice exposed to UVB. In the UVB-induced model, epidermal thickness was increased and the papillary dermis indicated amplified inflammatory cell infiltration, but the histopathological study in treated groups with *Morinda citrifolia* exhibited a significant reduction of inflammatory contents [42]. Histopathological findings in this study showed that amelioration of dermis and epidermis thickness, and also, collagen arrangement were observed in the ASC group while the improvement process of skin models was detected in the control and betamethasone groups. But also, the changes in inflammatory cells infiltration, thickness, and collagen regulation were more significant on day 7 in the ASC group. ASC group results suggested that anti-inflammatory effects after UVB-induced damages due to the reduction of inflammatory cells and polymorphonuclear leukocytes infiltration. Skin thickness decline was observed in histopathological evaluation. Moreover, the ASC group could increase collagen synthesis and alleviate collagen degradation on day 7, which plays an important role in wrinkle formation and photoaging procedures with reduction of skin thickness.

5. Conclusion

This study demonstrated that topical application based on ASC extract could improve the repair phase in the epidermis and dermis layer with skin barrier functions increment after UVB-induced damage. Also, it could decrease the thickness of the epidermis and dermis in the dorsal skin of animal models. The results indicate that the polymorphonuclear leukocytes and inflammatory cell infiltration were reduced during the 7 days of the treatment period. Therefore, the ASC extract showed that the anti-inflammatory and rejuvenating effects were related to anti-inflammatory mechanisms. Based on these findings, ASC extract is an appropriate candidate for clinical studies and is a part of UVB-induced damage therapy.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

D.K. and M.N. contributed equally to this work. The authors declare that the persons named in this article did this work. D.K. and H.A.A. were involved in study design, manuscript preparation, and data acquisition. D.K. wrote the manuscript and performed the data analysis. M.N. and F.H also contributed to data acquisition. H.A.A, F.H., and M.N. critically reviewed the data and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Research Article

In Vitro Comparison of the Effect of Three Types of Heat-Curing Acrylic Resins on the Amount of Formaldehyde and Monomer Release as well as Biocompatibility

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Background/Purpose. The biocompatibility and cytotoxicity of formaldehyde and monomer are essential in resin-based denture's byproducts. This present study was performed to compare the release of formaldehyde and monomer and biocompatibility of three brands of heat-curing acrylic resins, including Ivoclar, Bayer, and Acropars, with different mixing properties and the same processing methods. *Materials and Methods*. In this experimental in vitro study, 18 samples were fabricated from Ivoclar, Bayer, and Acropars heat-curing acrylic resins (each group consisting of 6 samples). The released formaldehyde and monomer level were measured and registered for 1, 7, and 30 days. Also, methyl methacrylate release from samples was used to test cell cytotoxicity using L-929 murine fibroblast. The data were analyzed with repeated measures, one-way ANOVA, and Kruskal–Wallis tests. *Results*. For formaldehyde release of 1 day, Ivoclar acrylic resin showed the lowest level, followed by Bayer and Acropars acrylic resins ($P < 0.05$). On 7 and 30 days, Bayer acrylic resin released the lowest formaldehyde, followed by Ivoclar and Acropars acrylic resins ($P < 0.05$). Acropars showed the weakest and most significant results regarding biocompatibility and monomer release in all three points of time, respectively ($P < 0.05$). *Conclusion*. Acropars acrylic resin showed the most significant formaldehyde and monomer release and least biocompatibility compared to Bayer and Ivoclar for 1, 7, and 30 days; however, after 30 days, all three resins displayed the same amount of formaldehyde release.

1. Introduction

Over the last decade, advances in dental material science have promoted the expectations of dentists and patients about the safety and efficiency of dental products [1]. Nowadays, there are several kinds of acrylic resins in the dental market, and each of them shows some advantages and disadvantages [2]. Most denture bases are made from heat-cured acrylic resins [3]. Polymethyl methacrylate (MMA) resin has been widely used to manufacture many dental materials for a long time [2]. On the contrary, many research studies have shown that heat-polymerized

acrylates have many advantages over cold-polymerized materials due to almost complete polymerization and better biological properties [3, 4]. One of the significant concerns about using MMA resins for denture base fabrication is the release of byproducts such as formaldehyde and monomer into the oral environment [5]. The residual monomer is the primary substance eluting from the acrylate denture base [6].

On the contrary, formaldehyde is formed as a product of oxidation of the residual MMA in the layers with a low degree of conversion [7, 8]. Investigations have shown a correlation between formaldehyde release and residual free monomer after polymerization [7, 9]. Various factors could affect the content of residual monomer including polymerization temperature and duration, the thickness of acrylic resin [10, 11], the measurement method [12, 13], length of the polymerization cycle, and the type of acrylic resin [12, 14]. Some studies have shown the lack of biocompatibility and cytotoxicity of this material [11, 12]. MMA monomer is an allergen and a contributing factor in the sick building syndrome (SBS), and even some studies of humans have suggested that MMA exposure is linked with certain types of cancers [4]. Long-term exposure to this substance causes a headache, burning eyes, and mutational consequence respiratory system irritation [2, 3, 5, 8]. According to the International Agency for Research on Cancer, formaldehyde has been introduced as a potential carcinogen for animals, but the carcinogenic effect of formaldehyde on humans is controversial [4]. However, the toxicity effects of formaldehyde on man and animals in some studies have been reported [13, 14]. Formaldehyde is cytotoxic in smaller quantities in comparison with the MMA monomer. It can irritate the mucosa even at low concentrations of 0.63–1.2 ng/ml [15, 16]. MMA molecules can be associated with saliva's proteins making large molecules responsible for oral tissue allergic reactions [17, 18]. Cheilitis, stomatitis, burning mouth, and painful sensation are some side effects of resin acrylic materials reported in people who have received a complete or partial denture [19]. All cells in the human body contain some amount of formaldehyde, which is produced due to the metabolism of serine, glycine, methionine, and other amino acids in the human body [4]. In the recent years, Ivoclar, Bayer, and Acropars heat-curing acrylic resins have been introduced for fabrication of removable prostheses in the market. The present study was conducted with the aim of comparing the effect of the three types of heat-curing acrylic resins on the amount of formaldehyde and monomer release as well as biocompatibility within 30 days.

2. Materials and Methods

In this in vitro study, all samples were fabricated using the conventional flasking method for denture base fabrication from Ivoclar (Ivoclar Vivadent, Schaan, Liechtenstein), Bayer (Meliodent; Bayer Dental Ltd., Newbury, UK), and Acropars (Marlic, Tehran, Iran) heat-curing acrylic resins, according to the manufacturer's instructions (Table 1).

2.1. Assessment of the Release of Formaldehyde

2.1.1. Specimen Preparation. Thirty cubic samples with $3 \times 3 \times 2\,\text{mm}^3$ dimension of each brand were provided and divided into three groups of 10 samples and weighted with a digital scale with 0.05 accuracy. The samples were incubated at 37°C and 100% humidity in 100 ml of double-distilled water in 3 closed glass containers. Assessment of formaldehyde release and calibration curve preparation was performed by Gigante et al.'s method [20]. In brief, 1 ml of formaldehyde solution (Merck KGaA, Darmstadt, Germany) with predetermined concentrations was poured in a 50 cc glass beaker. 300 microliters of chromotropic acid 5% (Sigma-Aldrich, Germany) was added to the solution with 3 ml phosphoric acid (H_3PO_4) 85% and 70 microliters of hydrogen peroxide (H_2O_2) 2.5% (Scharlau Chemie S.A., Spain) simultaneously. The glass beaker was covered with a glass block and was heated in an electric microwave oven with 1100 W power for 35 seconds. Afterward, the solution was cooled gradually at room temperature (25°C). Finally, the level of absorbance of the solution at the wavelength of 570 nm was measured using the UV-Vis spectrophotometer (Agilent Cary 60, USA) to provide the calibration curve. 1 ml of water in each closed glass jar was picked up. The molar concentrations of formaldehyde in distilled water were assessed using the previously described method according to the calibration curve for 1, 7, and 30 days. The remaining water was discarded and replaced with fresh distilled water each time.

2.2. Assessment of the Release of the MMA Monomer

2.2.1. Specimen Preparation. A total of 30 cubic samples measuring 10×50 mm with 1.5 mm thickness fabricated each denture base acrylic resins' brand. The samples were weighted with a digital scale with 0.05 accuracy, placed in closed glass containers containing 100 mL of double-distilled water, and incubated at 37°C and 100% humidity. To assess monomer release and preparation of the calibration curve, 1 ml of MMA monomer solution (Merck KGaA, Darmstadt, Germany) with predetermined concentrations was prepared, and the level of absorbance of the solution at the wavelength of 240.5 nm was measured using the UV-Vis spectrophotometer (Agilent Cary 60, USA) to provide the calibration curve. 1 ml of water of each container, as mentioned above, was collected for 1, 7, and 30 days after incubation, and the remaining water was discarded and replaced with fresh distilled water each time. The collected water was read in the spectrophotometer. The numerical absorbance values were recorded, and the corresponding concentration was extracted from the calibration curve and reported in μg/mL.

2.3. Assessment of Cell Viability (MTT Assay). Thirty cubic samples with $3 \times 3 \times 2\,\text{mm}^3$ dimension of each brand were provided and divided into three groups of 10 samples and weighted with a digital scale with 0.001 accuracies. To perform the MTT assay for each time interval (1, 3, and 7

Table 1: Physical properties of three brands of heat-cured acrylic resins.

	Water absorption (μg/mm^3)	Powder/liquid ratio	Processing method	Methyl methacrylate content (%)
Ivoclar	21.6	20.5 g/10 ml	Boiling water 35 min	95.9
Bayer	23.2	35 g/14 ml	Boiling water 20 min	>90
Acropars	30	24 g/10 ml	Boiling water 30 min	>90

days), ten samples of each brand were placed in a well (in a 96-well plate) in direct contact with L-929 fibroblasts (20,000 cells in each well). The samples and medium on cells were discarded at the test time intervals, washed with PBS, and replaced by the MTT-containing medium. Then, the 96-well plate was incubated for 4 h. To solve the formazan crystals reduced from tetrazole by viable cells, the MTT solution was discarded, and without washing, DMSO was added. The plates were left for 20 min and then transferred to the ELISA reader to measure the absorbance at 570 nm. The results were expressed as a percent of viable cells according to positive and negative control groups.

2.4. Statistical Analysis. The data were analyzed with repeated measures, one-way ANOVA, and Kruskal–Wallis tests. The data were statistically analyzed using SPSS version 22, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Formaldehyde Release. The formaldehyde release (mol/L) level was assessed according to follow-up intervals of a day, a week, and a month (Figure 1). Statistical analysis by the two-way repeated-measure ANOVA revealed a significant difference between Acropars and two other groups considering the effect of time and resin on formaldehyde release for 1 and 7 days ($P < 0.05$). However, the results failed to reveal any significant effect of time and type of resin after 30 days ($P > 0.05$). One-way ANOVA showed that Acropars resin exhibited the highest level of formaldehyde release for 1, 7, and 30 days. The difference of Acropars formaldehyde release compared to Bayer and Ivoclar was significant ($P < 0.05$) for 1 and 7 days, while the difference between 3 groups was not significant ($P > 0.05$) for 30 days. Also, no significant difference was recognized between Ivoclar and Bayer resins for all intervals ($P > 0.05$). During the 30 days of the experiment, formaldehyde release lasted from all three acrylic resins; however, it decreased significantly compared to the first day after processing the resin.

3.2. Monomer Release. During 30 days, the highest and the lowest amount of monomer were released from Acropars and Bayer, respectively (Figure 2). According to ANOVA, this difference was statistically significant ($P < 0.05$), while the difference between Bayer and Ivoclar was not significant in three points of time ($P = 0.8$, $P = 0.6$, and $P = 0.8$).

3.3. Cell Viability (MTT Assay). The cell viability results at different time points are presented in Figure 3. The highest percentage of cell viability for 1, 3, and 7 days belonged to Ivoclar and Bayer, while the lowest was reported in Acropars resin compared to the control group. According to the Kruskal–Wallis test, this difference was statistically significant ($P < 0.05$). On the contrary, no significant difference was noted between Bayer and Ivoclar acrylic resins compared to the control group during one week.

4. Discussion

In our study, the amount of monomer and formaldehyde release and cell viability were investigated in three brands of heat-curing resins, which made our study unique and comprehensive compared to other studies conducted in this field. To the best of our knowledge, no article in this field evaluated these three features simultaneously in acrylic resin materials. Biocompatibility and cytotoxicity of dental materials should be taken care of when materials are used to fabricate dental applications to prevent their toxic effect on the surrounding tissue. Based on previous studies which recommended heat-curing resins as the lowest harmful dental material, three popular heat-curing acrylate resin brands in the dental market were chosen to investigate in this study [15, 18, 21].

The present study showed that the difference in formaldehyde release from Acropars acrylic resin was significant compared to Bayer and Ivoclar acrylic resins for 1 and 7 days, but the difference among these three groups was not statistically significant for 30 days. Furthermore, Acropars monomer release was statistically significant all the time compared to Bayer and Ivoclar.

Formaldehyde is one of the certain toxic chemicals, which is released gradually from acrylic resins resulting in irritation of the surrounding tissue [11]. It is one of the byproducts generated due to the oxidation of methacrylic groups or degradation of the oxide-methacrylic copolymer [22]. MMA concentration and polymerization rate play an essential role in releasing formaldehyde from acrylic resin materials, one of the many factors responsible for cytotoxicity and irritating effects of acrylic resins [10]. As shown in our study, there was a direct relationship between MMA and formaldehyde released from the three heat-cured acrylate resins tested. It means how much the leaching of MMA rises, and formaldehyde will enhance directly. This finding was by other previous studies [10, 21].

Tsuchiya et al. [21] assessed the amount of leaching and cytotoxicity of formaldehyde and methyl methacrylate monomer from three denture base resins into saliva, including autoresinizing, heat-curing, and microwave resins, for the first day until one month later. They observed that the level of these substances in saliva was high for the first day, and the leaching continued for one month; however, it decreased gradually. Also, the level of cytotoxicity of formaldehyde was higher than that of methyl methacrylate,

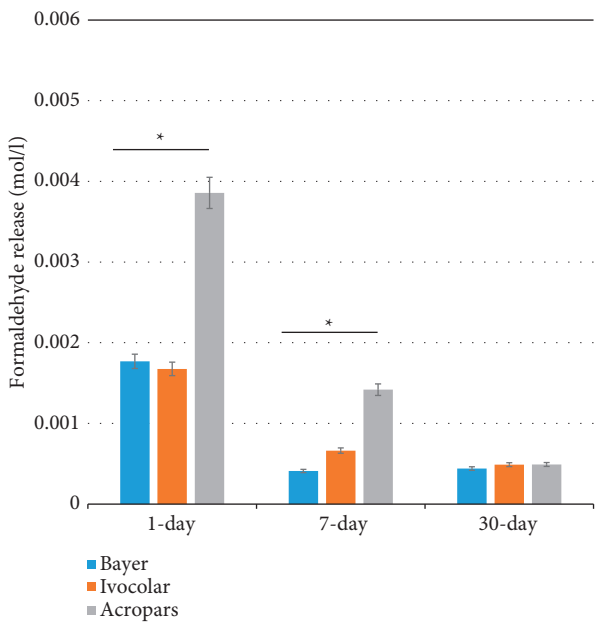

Figure 1: The amount of formaldehyde release (mol/L) is categorized by the type of acrylic resin according to follow-up intervals.

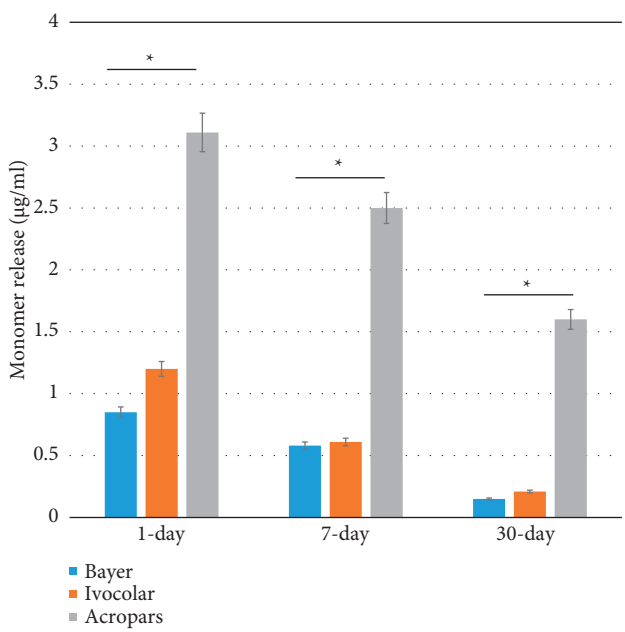

Figure 2: The amount of monomer release (μg/ml) is categorized by the type of acrylic resin.

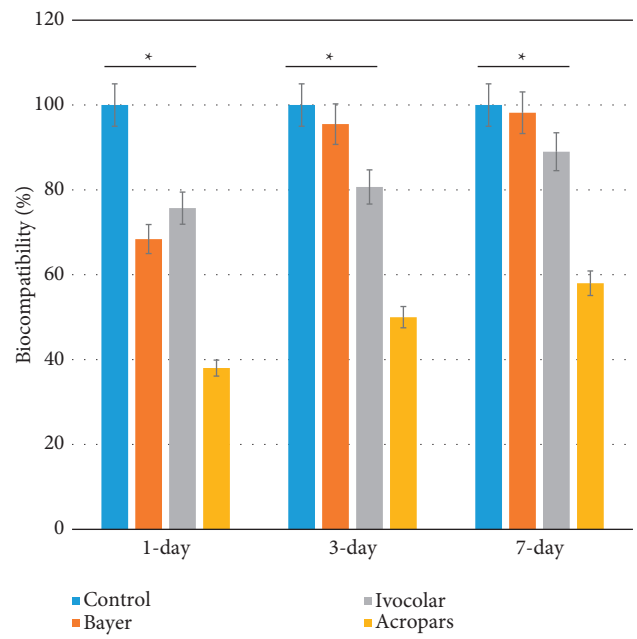

Figure 3: Amount of biocompatibility (%) according to follow-up intervals and categorized by acrylic resin type.

even at lower concentrations. Furthermore, in heat-curing resins, the amount of leaching of the mentioned substances was significantly lower than other forms of acrylic resin polymerizations. Cytotoxicity might be related to the amount of formaldehyde released from resins, which was reduced over time [22]. In the present study, it was observed that, during the 30 days of the experiment, formaldehyde and monomer release lasted from all three heat-curing resins; however, it decreased significantly in comparison with the first day after acryl processing that confirmed Tsuchiya findings. In 2012, Kostic et al. [23] assessed the cytotoxicity of three groups of denture base acrylic resins, including soft and hard autopolymerizing and heat-curing resins, by using a direct contact test. They concluded that autopolymerizing acrylic resins (soft or hard) had a mild inhibitory effect on cell growth. On the contrary, heat-curing acryl exhibited no signs of cell growth inhibition. Since the cell growth inhibition effect of acrylic resins was attributed to the monomer and formaldehyde released as byproducts of the acrylic resins [18], it was concluded that the heat-cured resin showed the smallest amount of formaldehyde and monomer release compared to other acrylic resins. In contrast, in our study, we concluded that even heat-cured acrylic resins could produce the noticeable amount of formaldehyde and monomer resulting in cytotoxicity of these materials. Thus, it seems that more research through heat-cured resins is necessary in order to find the most biocompatible materials. Ebrahimi Saravi et al. [24] in 2012 assessed the biocompatibility of three acrylic polymers by the use of L-929 murine fibroblasts, and the MTT assay revealed the superior biocompatibility of Bayer to Futura Gen and GC Reline at one hour, 24 hours, and one week which was similar to our findings. The present study chose three different heat-cured resins to determine the least harmful heat-cured acrylic resins. Formaldehyde and monomer release from Acropars acrylic resin for 1 and 7 days was beyond the permissible limit declared by the official website of the Iran Food and Drug Administration, which is under supervision of the Ministry of Health which reached the limit for 30 days. Besides, MTT assay revealed that Acropars and Bayer acrylate resins showed the least and most biocompatibility, respectively. On the contrary, in our study, like many previous studies, the MTT cytotoxicity test was used to investigate the toxicity of MMA. It has been

shown that MMA was not the only substance released from acrylic denture bases. Other substances could be detected, including methacrylic acid, benzoic acid, phthalates dibutylphthalate, dicyclohexyl phthalate, phenyl benzoate, and phenyl salicylate, as well as formaldehyde. Also, Sadamori et al. [25] reported that residual monomer content in acrylic dentures could be detected for up to several years after use. It appeared that complete loss of the residual monomer content might take more than 30 days that we have detected. Hence, more research in this field is mandatory either to evaluate the amount of monomer release for a longer time or to detect other cytotoxic substances released from the denture base. Sheridan et al. [26] reported that, during the first 24 hours, the cytotoxic effect of acrylic resins, which was associated with the rate of polymerizations and chemical composition of the resin, was greater and moderated after a week. Our study resembling Sheridan et al.'s report showed that the peak formaldehyde release, which was one of the most important factors of cytotoxicity, was observed during the first 24 hours and subsided over time. Except for Tsuchiya et al. [21], who determined formaldehyde release similar to the present study, other researchers have evaluated just free methyl methacrylate monomer [19, 22, 24, 27]. However, formaldehyde is more cytotoxic than methyl methacrylate, even at lower concentrations [21]. Therefore, substances with the probability of high formaldehyde release should not be used. Also, since formaldehyde is formed due to the oxidation of free monomer mostly at the superficial layers [28], acrylic resins should be carefully processed and immersed in water for 60 minutes after deflasking the level of free monomer and formaldehyde [29]. For many years, heat-cured acrylic resins have been the most commonly used denture base materials [29, 30]. Still, it is preferable to choose a type that releases the least formaldehyde and monomer due to its adverse effects on the oral mucosa. Using Acropars, acrylic resin showed the greatest formaldehyde and monomer release for all intervals compared to Bayer and Ivoclar acrylic resins. Also, Acropars acrylic resin was less compatible than Bayer and Ivoclar acrylic resins. On the contrary, the difference between Bayer and Ivoclar acrylic resins was not significant at 30-day intervals; however, Bayer displayed the fewer formaldehyde release.

Data Availability

All the data generated or analyzed during this study are included within this published article, and also, the datasets analyzed to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Review Article

Free-Hand versus Surgical Guide Implant Placement

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One of the most key areas of dentistry is dental implant surgery. The use of digital equipment and software in dentistry has developed considerably in recent years compared to other fields of medicine. Since examining the advantages and disadvantages of each approach, along with case studies, can help physicians make informed decisions, this review study aims to raise the awareness of dentists to make easier decisions about using guided or free-hand surgery. When planning for a dental implant, one of the most challenging questions that doctors face is which method to use (guided surgery or free-hand). Choosing the right method, such as other clinical considerations, will depend on the individual circumstances of each patient and the preference of the treating physician. Free-hand surgery is a cost-effective method in which the flap is reflected, and, according to the doctor's diagnostic information, an implant is placed, which in many cases is a useful method. Guided surgery has the highest level of accuracy and control, in which osteotomy is designed and printed through a digital surgery guide, and depending on the complexity of the case and the patient's anatomy, it has a higher level of value than free surgery. The surgical guide helps the surgeon make the implant surgery more accurate, safer, simpler, at a lower cost, and in less time. In fact, there are patterns that convey information about the position of the tooth to the dentist before the implant is placed.

1. Introduction

Dental implants are performed when a person has lost a tooth for any reason, and the implant is used to fill in the gaps [1]. One of the key factors that make implants known as a reliable option is successful osseointegration, which requires a method that achieves minimized surgical complications such as nerve damage, perforation, and cortical plate perforation to achieve this goal and ultimately achieve the desired result [2]. One of the key factors that make implants known as a reliable option is successful osseointegration, which requires a method that achieves minimized surgical complications such as nerve damage, perforation, and cortical plate perforation to achieve this goal and ultimately achieve the desired result [3]. The most common clinical-pathological findings associated with dental implants are hard tissue defects [4], such as defects at implant sites encompass intra-alveolar [5], dehiscence, fenestration, horizontal ridge [6], and vertical ridge defects and soft-tissue defects include volume and quality deficiencies with a lack of keratinized tissue [7], which can lead to marginal bone loss, soft-tissue inflammation, and soft-tissue stagnation [8–10].

Since the introduction of modern implantology to the medical community in the early 1980s, surgeons have always sought to place implants in terms of the amount of bone left in the patient's jaw [11]. This sometimes causes the implants to be placed in the wrong direction inside the jaw and, in many cases, makes it difficult or impossible to achieve a

proper prosthesis, both aesthetically and functionally [12]. It is important to understand that surgeons usually tend to place the implants in the largest volume of bone left, but in most cases, this shape of the implant has caused the buccal or lingual position of the implant to be too much, and it provides problems for prosthodontists and laboratory technicians in preparing prostheses as efficiently and beautifully as possible [13]. Unfortunately, such problems remain hidden from the surgeon and patient until the implant is cast, and the prosthodontist uses very expensive equipment and unusual prostheses to treat the patient, which, although it leads to the preparation of prostheses, however, in the end, despite all the efforts of the prosthetic staff, is inefficient and ugly for the patient and sometimes causes legal issues which, of course, pleases no one [14]. In this regard, the use of new technologies and modern software provides the possibility of three-dimensional examination of the location of implants, making the diagnosis and treatment of patients more reliable [15].

Dental implants are performed in two general ways, which are implantation with the normal method and implantation with the surgical guide. The surgical guide allows dental implants to be performed in the most accurate location and with the least amount of surgery. The correct position of the implant allows the optimal design of the definitive prosthesis and prevents the possibility of cemented repairs by making it possible to design and manufacture recyclable screw prostheses [10, 16]. From the patient's point of view, guided surgical procedures are no different from conventional surgery, and only an additional scan/image is taken of the patient's mouth. The procedure is that the teeth scan and CBCT of the patient's jaw are merged together in the guide design software [17]. As a result, the nerves of the jaw, sinuses, the roots of adjacent teeth, and bone density are examined before each operation, and the implant is placed virtually in the best location [18].

Based on this design, a surgical guide is made and sent to the treating dentist. It is even possible for all veneers to be sent to the dentist before the patient visits. Therefore, the dentist places the implant in the same space as specified in software without stress and, more importantly, without error [4]. As a result, the patient in one session with the lowest risk of infection will pass the implantation stages without surgery and with the lowest risks [19]. In some cases, a combination of surgery and a surgical guide is used as directed by a physician. If the implant can be placed in the best place and in the best conditions, it will lead to a long life of the prosthesis [20]. Despite the fact that different studies have introduced different types of these surgical guides, it does not seem to be any strong consensus either on classification or on defining different types of surgical guides [21, 22]; therefore, the aim of this review study was to gather available data in regard to the classification and the whole idea of practicing implant dentistry using guided surgery.

2. Free-Hand Surgery vs. Guided Surgery

In free-hand surgery, panoramic and periapical radiographs are used to assess the width and alveolar bone profile available to place the implant and examine the surrounding anatomy and ultimately rely on CBCT imaging. In this method, periodontal probes, gauges, or calipers are applied through the intraoral exam to make a sound in the bone, which gives a logical view of the height and thickness of the ridge [23]. The surrounding teeth can also be used as a guide for determining the correct position of the implant. It should be noted that the implant should be at least 1.5 mm from each adjacent tooth and 2 mm apical to their enamel cementoenamel junction [13].

Implants that are placed too close to the root of an adjacent tooth can result from poor surgical technique, poor treatment planning, insufficient space, and incorrect angle, which can damage the periodontal ligament and surrounding structures. This can lead to bone displacement in the periodontal ligament (PDL) space, which ultimately leads to changes in blood supply to adjacent teeth, loss of tooth freshness, apical periodontitis, and internal or external resorption [24].

Implants very close to nearby teeth are more likely to be lost due to infection or bone resorption. If the distance between the implant is more than 1.5 mm to the adjacent tooth, any bone defect around the implant remains a vertical defect which in most cases; the bone loss does not occur in adjacent natural teeth, and if this distance is less than 1.5 mm, then the bone on the adjacent tooth will maintain the height of the interdental papilla. In any case, not observing enough space between the tooth and the implant can lead to irreparable damage to adjacent teeth and their decay and fracture [13].

Free-hand surgery has many benefits for the dentist because it can visualize and relate diagnostic data to the actual clinical condition by reflecting soft tissues and examining bone anatomy [25]. Additional treatments such as bone grafts, PRF, and GBR can be easily performed if needed. On the contrary, the surgeon is able to make measurements through diagnostic casts, and making a diagnostic replica model gains a better understanding of the mesial-distal space and the apico-coronal space in order to determine the exact location of the implant on adjacent teeth by the prosthesis [26]. The use of diagnostic wax will also help in planning the surgical procedure, which will result in the implant supporting the final prosthesis in the best possible way. In free-hand surgery, the bone beneath the implant can be directly evaluated during surgery and measured with bone calipers after opening the flap [23].

The procedure for guided implant surgery is different from free-hand implant surgery. In guided implant surgery, after completing the CBCT imaging, a DICOM file is created, which is accompanied by a digital intraoral impression or precise putty light body impression to prepare the model. The DICOM file is passed to the implant scheduling software along with the patient data. This software uses DICOM file to present data in two dimensions and three dimensions [10, 27]. Figure 1 demonstrates conventional and digital workflow as a chart [28].

Software allows the user to visualize important anatomical milestones such as the nasopalatine canal, maxillary sinus, inferior alveolar canal, and submandibular fossa, which is an auxiliary factor in implant position planning using different tools [29].

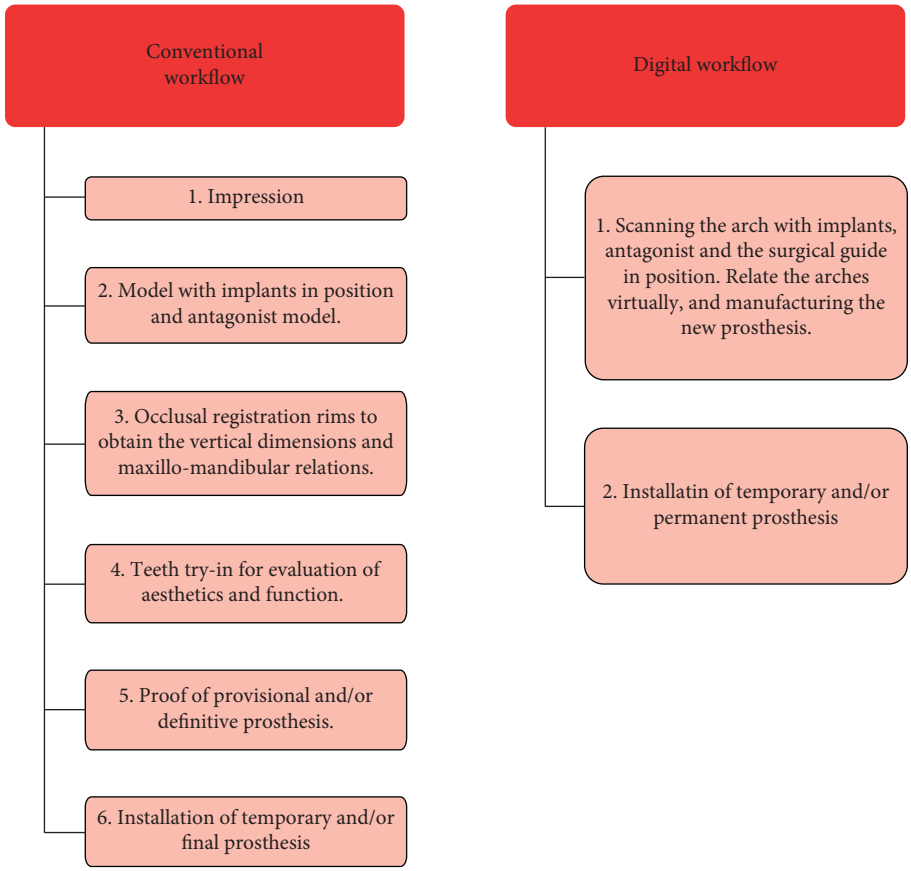


Figure 1: Conventional versus digital workflow in implantology [28].

After completing the treatment planning, the obtained digital information is included in the stereotyped surgery guide and becomes physically available. The surgery guide pattern can be designed differently depending on whether the patient has teeth or is completely toothless, depending on the number of implants to be implanted. For example, in patients whose teeth are preserved around the implant site, the surgical guide pattern is used to maintain stability [30]. In toothless people, the pattern has horizontal sleeves for anchor pins to be fixed in the patient's mouth [31].

This guide has round metal sleeves made of titanium or other alloys at the implant site that the depth, angulation, and mesial-distal and buccal-lingual locations of the implant are precisely controlled by these titanium sleeves [32]. Each surgery guide is made according to the guided implant surgery kit. The drills in this kit are designed to be suitable for osteotomy and suitable to perform it. Each of any drills has a stopper that rests on the occlusal exterior of the lip of the metal sleeves. The length of the metal sleeves determines the distance between the neck of the implant and the occlusal surface of the sleeves (offset) [31].

3. Classification of Surgical Guides

There are different opinions about the classification of several types of surgical guides. For example, Balshi and Garver [33] consider the condition of the patient's teeth as the main parameter and introduce three basic surgical guide stents for implant placement.

(1) Completely edentulous (supplies a general guide to the area of dental implant positioning and a particular guide to the location and angulation that of each dental implant have need of placement) (Figure 2) [34]

(2) Slightly edentulous/removable partial denture design

(3) Slightly edentulous tooth-supported design (Figure 3) [35]

Considering the remaining dentition and needed accuracy for the guide, there have proposed 4 options in regard to supporting area: tooth supported, tissue supported, tooth and tissue supported, and tissue supported with an accessory fixation for edentulous patients [36].

A conceptual method is also used to describe different types of surgical guides, which includes three different concepts [24, 37]:

(1) Nonlimiting design

(2) Partially limiting design

(3) Completely limiting design

These three concepts are classified based on the amount of limitation that surgical guide templates offer [37].

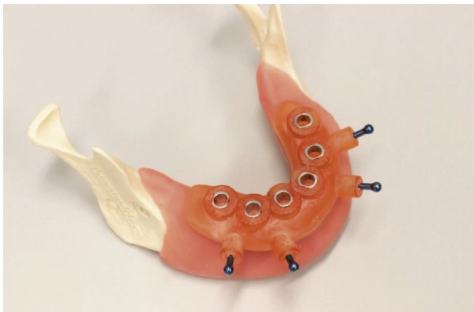
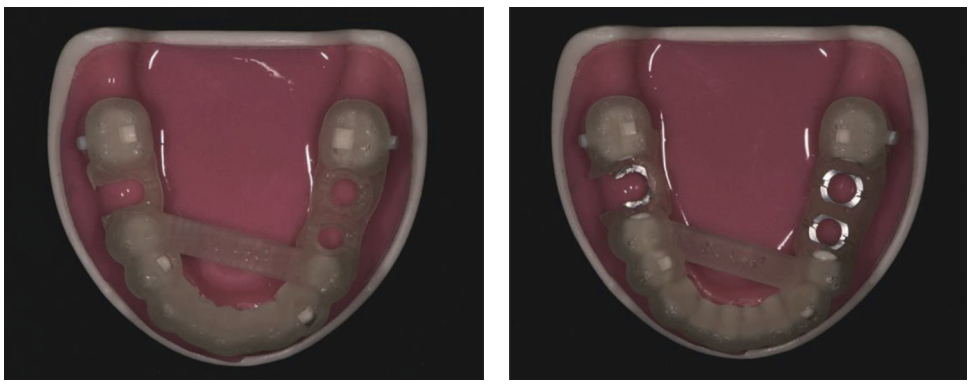


Figure 2: Printed surgical guide for a full mouth rehabilitation using four guide pins [34].



(a) (b)

Figure 3: (a) Metal sleeve-free surgical guide. (b) Metal sleeve incorporated surgical guide [35].

The first one is a simple, unrestricted surgical guide, so-called free guide [36], that tells the operator where to go in relation to the implant site and guide the surgeon on where the future prosthesis would be in relation to the implant, and the operator will decide on other parameters related to the exact angle and position of the implant. It also provides the surgeon with the best location of the implants without too much focus on the angulation of the surgical drill, therefore allowing too much flexibility; the operator will decide on other parameters related to the exact angle and final position of the implant. In this regard, a technique was described in studies [38, 39] in which a guide pin hole is fabricated by drilling through a clear vacuum-formed matrix. This hole functioned as the best position of the implant and adjacent and opposing teeth were used as a guide to determine the angulation. Therefore, these templates could be served as imaging indicators in the implant surgical phase.

The second type of surgical guide known as access guide [36] is somewhat restrictive, which is a guide sleeve that will direct only the first drill used for the preparation of the implant site there, and other preparation steps are done by the surgeon [37].

As the last concept, the completely restrictive surgical guide or the precision guide [36] wherein all the instruments used for drilling the implant area by that guide, containing the buccolingual and mesiodistal planes, as well as to drill, stops limiting the depth of the preparation and so the final positioning of the prosthetic part [37]. Since these guides are more restrictive, decision-making and following surgical procedure that is done during the operation would be less. This concept has 2 common designs: computer-assisted design and manufacturing (CAD/CAM)-based surgical guide and cast-based guided surgical guides.

Considering 3 design concepts and 4 supporting area mentioned before, there could be 12 types of surgical guides [36]. Requiring a free or access guide with tissue support and accessory fixation would be somehow rare; therefore, feasible options for a surgical guide would be as follows:

(1) Nonlimiting, tooth supported
(2) Nonlimiting, tooth and tissue supported
(3) Nonlimiting, tissue supported
(4) Partially limiting, tooth supported
(5) Partially limiting, tooth, and tissue supported
(6) Partially limiting, tissue supported
(7) Completely limiting, tooth supported
(8) Completely limiting, tooth, and tissue supported
(9) Completely limiting, tissue supported
(10) Completely limiting, tissue supported with accessory fixation

4. The Effect of Surgical Guide Pattern on Implant Accuracy

Accurate placement of the implant is critical to achieving an aesthetic result and correct alignment to withstand occlusal

forces for long-term success [40]. Despite the popularity of the surgical guide model in the clinic, there is still disagreement about its effect on the accuracy of implant placement. Some researchers believe that implant placement using a surgical guide is more accurate than other methods [23]. Others have argued that despite the high accuracy of dental implants when using the surgical guide, free-hand implant surgery accuracy has been sufficient and acceptable for most clinical conditions [8].

In their study, Wang et al. showed that the use of the surgical guide makes a significant difference between the planned and actual positions of the implant, especially at the implant shoulder, root apex, and angulation relative to the manual implantation method [41]. It has been clearly proven that the placement of dental implants using surgical guides is more accurate than implants that are placed without a guide [42, 43]. High accuracy in implant placement is of great clinical importance and has several benefits. Among these benefits is its safety. Preoperative planning using surgical guides can ensure the safety of implant placement and reduce the incidence of complications, which is an important advantage for young and inexperienced surgeons and makes implant surgery easier for them. Also, when planning for a surgical guide, it is easy to evaluate that the proper angulation and occlusal relationships are more readily assessable using dental casts where the lingual aspect is not obscured [44, 45].

5. Evaluating the Effect of Accuracy Factors on the Position and Angle of the Implant

Determining the exact position of the implant in the bone is often difficult due to the location of the implant and its angle [46, 47]. Physician experience, tooth-born status, timing relative to extraction, and the number of adjacent implants are identified as the four major factors influencing implant position. Also, tooth-borne status, number of adjacent implants, and the width of the edentulous space for the subset of tooth-borne, single-implant cases, have been introduced as three main influential factors on implant angulation [13].

It has been observed that the number of implants that are to be placed in proximity has a significant effect on the position of the implant and its angle. As mentioned, single-implant cases are more accurate than cases where 2 or 3 implants are placed side by side [48]. Also, the angle and position of implants placed in people who have teeth are more accurate than in people who are completely toothless. Among the factors influencing accuracy, the presence of adjacent teeth on both sides of the implant has the greatest impact on the accuracy of position. The location of the extracted tooth is also one of the factors affecting the accuracy of the implant angle, but its impact is less than the impact of the number of adjacent implants [49].

Another influential factor is the time of implant placement relative to the time of tooth extraction. Delayed implants (time interval between tooth extraction to implant placement) have been observed to be significantly less accurate and the cases of immediate implants (implantation done on the same day as the tooth is extracted) are in a more precise position [50]. In terms of mesiodistal angulation between immediate and delayed cases, no significant statistics have been obtained [51]. The results of various studies show that immediate implants are in a more accurate mesiodistal position, but do not differ in angle [52].

The most severe angular differences in the lower-molar implant subset have been observed in cases with distinct radiographic lines from the previously extracted tooth. In summary incomplete, partial radiographic bone remodeling after extraction predicted higher positioning accuracy and a trend toward lower angulation accuracy. It is also reported that arch, location on the arch, and implant dimensions have little to do with planting accuracy. In general, the results of studies show that fully guided implants that use a surgical guide are more accurate than implants that use traditional surgical guides and free-hand surgeries [44, 52–54]. Figure 4 [34] depicts the accuracy obtained through guided surgery by superimposition of the digitally planned implants and the actual treatment.

6. The Rate of Failure Associated with Implant Placement by Surgical Guides versus Free-Hand

The amount of research carried out on the impact of the surgical guide on implant success and survival is limited. Criteria for implant success include peri-implant radiolucency, the absence of mobility, pain, and infection [55, 56]. It was stated that the the maximum annual bone loss after one year of implant placement should be less than 0.2 mm [55].

Implant survival can be tracked by examining its stability in post-implant examinations. It is stated that the standard success rate should be 85% after 5 years of implant placement and 80% after 10 years [57–60]. Primary implant failure, which is mainly due to improper planning or surgical complications, causes problems for patients and surgeons [61, 62]. Implanted sites that fail for the first time are less likely to survive and are more at risk if they are re-implanted [63, 64]. Therefore, efforts should be made to make the initial implant successful. The success and survival of the implant depends on the osseointegration between the surface of the implant and the bone around the implant site [65] so that the loss of bone formation will cause the implant to fail [66, 67]. Most of the initial implant failures occur before the prosthesis is loaded, which is seen in less than 5% of patients in the first few weeks [68, 69]. Late failures that can be due to factors, such as circumstances affecting the microbial environment, peri-implantitis, and prosthetic rehabilitation, occur most often after the prosthesis is loaded. These failures are seen in an average of 7% of patients [70–72].

In a study, Yogui examined the survival rate of implant placement in both surgical guides and free-hand methods. The results of his 5-year study on implant survival in patients show that both techniques have had similar results in terms of survival, with rates ranging from 91 to 100% [25]. In another systematic study, the rate of implant failure in free-

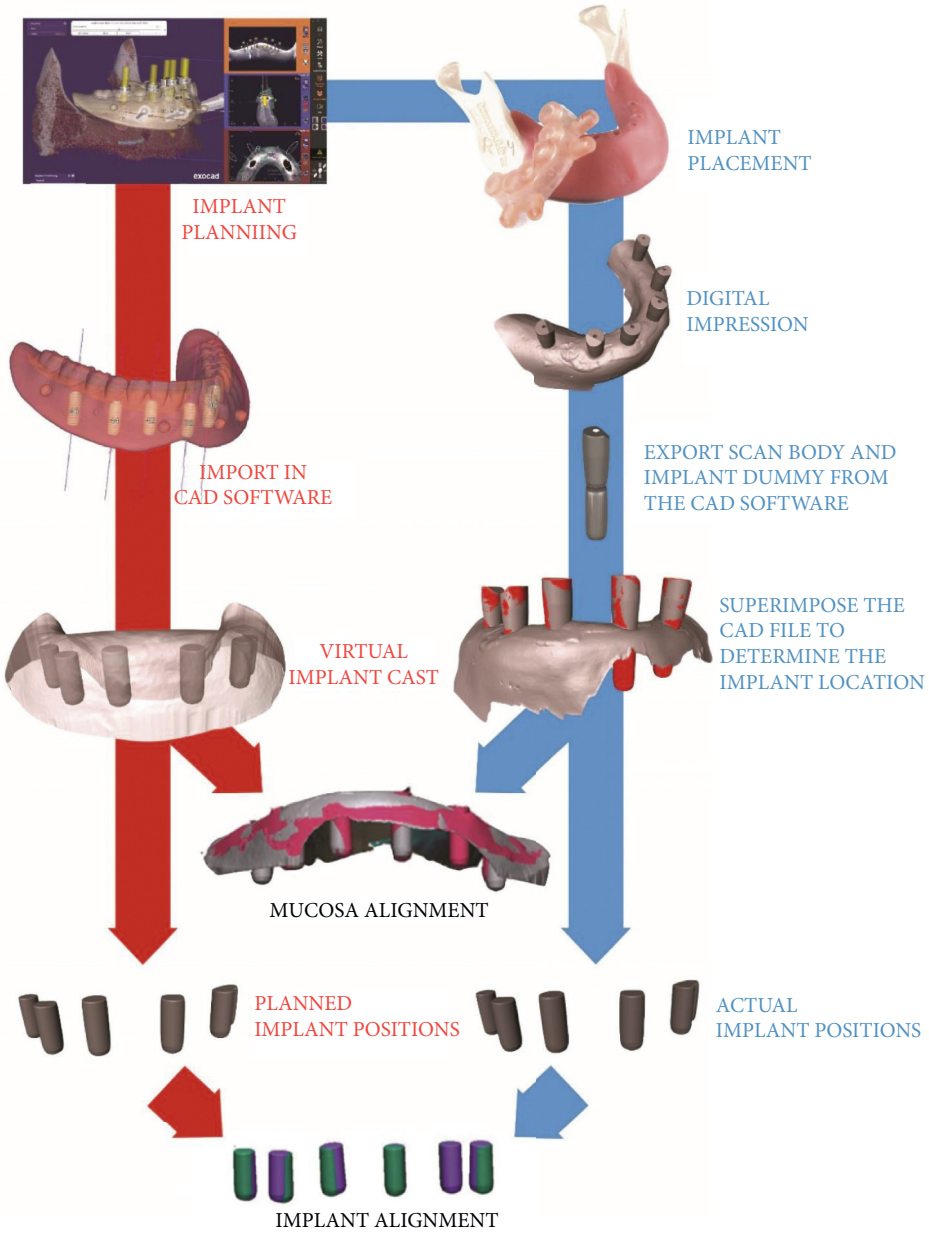


Figure 4: Actual and planned treatment plan superimposition regarding accuracy [34].

hand was reported to be almost three times that of surgical guides [73]. In addition to the type of implant implantation, factors which can increase the initial and late risk of implant failure include age and sex [74], smoking [56], tooth loss, bone quality and volume [75], implant site, diameter and length of the site [76], and, finally, the immune factor and various systemic diseases [56].

7. Complications and Possible Errors of Guided Implant Surgery

The risks of guided implant surgery are related to possible deviations between the position of the planned implant and the final clinical outcome. Accuracy can be achieved using specialized software. Accuracy check is possible with parameters such as deviation at the entry point, deviation at the apex, angle deviation or deviation of the long axis, and deviation in height/depth [6]. On an average, < 0.5 mm errors were reported from computed tomography or cone-beam computed tomography imaging acquisition and data processing [77, 78].

Recently, cone-beam computed tomography has been preferred to multilayer computed tomography for implant treatment planning due to its relatively low radiation dose, lower cost, high-resolution 3D images, and higher operating speed [79, 80]. Another cause of implant-guided surgery error is the patient's movement during the scan. Therefore, using an occlusal bite index to stabilize the mandible and scan the prosthesis, especially in patients who do not have teeth, will help to correct the error, and the scan should be repeated if necessary [81].

8. Results of Guided Implant Surgery Compared to Free-Hand Implant Placement

In various studies, successful use of guided implant surgery has been reported in people who have completely lost their teeth for any reason, along with the concomitant delivery of a prefabricated prosthesis for immediate replacement of missing teeth [82]. Nickenig et al. used guided implant surgery on patients who were partially edentulous, with 58% of the 250 implants performed on 102 patients without flaps. An implant which is planned had to be converted to a shorter one, and in four next circumstances, the limited interocclusal distance impersonated challenges while drilling. In eight cases, the guide could not be used, and implant placement required bone strengthening, which delayed implant placement. In nine of the implants, the final angle was different from the planned angle, with no clinical consequences [54].

In another study, which was performed comparatively between two groups of patients, it was observed that, in flapless patients, the duration of surgery, pain intensity and analgesia, and cases of trismus and bleeding were much lower than patients with free-hand surgery implants [83]. The results of several randomized controlled trials show that implant with guided surgery leads to greater accuracy, less pain and swelling, and shorter surgery time, but is more costly than free-hand implants. In these studies, no significant differences were reported between guided implant surgery and free-hand surgery in terms of implant success/failure or clinical parameters such as marginal bone loss [84–86].

In a prospective cohort study, the clinical performance of guided implant surgery was evaluated in comparison with the free-hand method, and it was reported that implants performed using the surgical guide method increase the accuracy of implant placement. Also, angular deviation was one of the most important parameters improved using this method compared to the free-hand method [22].

9. Advantages and Disadvantages of Free-Hand Surgery and Guided Surgery

The guided surgery has several advantages. Manual errors related to implant placement in this method are greatly reduced. Since, when using this method, the least intervention is done on the patient, therefore, problems after surgery are minimal and both the patient and the doctor will be calm in terms of psychological dimension [87]. Implants performed in this way are much more accurate and have higher safety, so the results of the implant will be predictable. From a hygienic point of view, because the implants are placed in the correct position, the oral health of this method is relatively guaranteed. This method greatly increases the survival of implant placement [88–90].

There are many benefits to using a surgery guide for the dentist; the surgery guide optimizes the location, angle, and depth of implants. One of the most serious complications of implant surgery that can be minimized with guided implant surgery is damage to important anatomical structures (sinuses, nerves, arteries, and teeth) [91]. It also provides the dentist with increased vision of the surgical site and easy access to flap exposure. This method will also be immensely helpful for beginners. Due to the reduction of implant surgery time and high success rate and no failure in this method, the overall cost is much lower than other methods. Surgical guide is the best method for full edentulous cases to place all implants in parallel and the best way to place multiple implants side by side in parallel. Avoiding the retraction of flaps and sutures will reduce postoperative pain, edema and bleeding, and immediate resumption of oral hygiene practices [92–94].

It may also be advantageous in comparison with conventional methods in patients with reduced bone quantity. In theory, the need for augmenting the residual bone may be eliminated or decreased by optimizing implant positioning in the available bone [95, 96]. On the opposite side of view, the available evidence lacks data regarding the application of digitally guided implant procedures to reconstruct resorbed edentulous ridges [97].

The application of an image-guided protocol in cases with severely resorbed posterior maxillae has been suggested as a proper alternative to insert implants in a restricted quantity of bone [98]. Considering the most recent systematic review studies [99–101], only the mentioned study was able to propose such a technique to propose the computer-assisted surgery as a substitute to bone regeneration techniques. Not all residual ridges could be managed accordingly and without considering reconstructing them. This is especially an important issue in patients with severe horizontal and vertical bone loss where anatomical structures could limit the final position of the implant [97].

The disadvantages of this method are several. Among other things, after making the guide, if necessary, it will not be allowed to change during the surgery. Also, if there is a change in the tissue between the time of ordering and the implant installation, it will change the fit of the prosthesis and ultimately the function of the implant prosthesis. If the guide is not stabilized and drilling is intended to penetrate hard bone, producing torsional forces on the sleeves, guide dislocation can occur during surgery. Also, in this method, there are costs related to purchasing software and special tools and drills, as well as spending time learning the curves for the treating physician [16, 89, 90, 102–104].

The advantages of free-hand implant placement include eliminating the time required to prepare the guide and reducing the cost of making the guide [25]. There are some limitations of free-hand implantation. First, clinical judgments about implant placement will be based on visualization of the clinical condition through information provided by cast and radiography. The second limitation is the longer time of this method than the surgical guide method because free-hand implant placement requires thinking and planning. Another limitation of the free-hand method is that aligning multiple implants using the free-hand technique is difficult, and the results are less predictable than surgical guides [13, 105]. Therefore, human error in this method will be much greater. Increased recovery time, swelling, pain, and bleeding are other disadvantages of the free-hand technique [13].

10. Conclusion

Dental implants have been a viable treatment option for patients with dental defects since the 1980s [106]. Dental implants have attracted the attention of patients and dentists due to the lack of damage to adjacent teeth and features such as comfort, high level of health, beauty, and long-term stability. However, observing such things as the correct depth of the implants is very necessary in performing the implant correctly. Today, with the development of techniques such as computer aided design and rapid prototyping, the surgical guide model has become increasingly used in dentistry. The use of a surgical pattern gives surgeons the chance to transfer the preoperative plan to the surgical procedure before taking any practical action on the patient, thereby both minimizing the invasion and shortening the operation time [106].

It seems that the surgical guide to some extent increases the accuracy of implant placement. Guided implant surgery can lead to errors that have been identified as the most common mistakes, such as misinterpretation of the tomographic image or misprocessing, deviation of 0.1 to 0.2 mm in the construction of the surgical guide, and incorrect fixation of the guide with the consequences of displacement during surgery [6]. The number of adjacent implants is a major predictor of mesiodistal position and angular accuracy. In contrast, tooth position and tooth extraction time are significantly related to position accuracy. The doctor's experience and the width of the edentulous space are also important factors in the position of implant placement and angulation, respectively [107].

In general, based on the results of clinical information, it can be concluded that, in people who have adjacent teeth and need an implant, a free-hand implant is a good option for them, but in contrast, for people who have no teeth at all and need to have multiple implants, proper guided surgery should be used. Narrow spaces less than 11 mm between teeth could be more suitable for free-hand implant placement in only one implant cases due to changes in position and lower angle changes. However, there is a possibility of approaching the root of the adjacent tooth and requires greater care. Because the placement of the implant immediately after tooth extraction improves the accuracy of the mesiodistal position. Therefore, using guided surgery over free-hand implant is preferred. It is also recommended that, in cases with incomplete bone regeneration and fallen roots, for example, in lower mill teeth, guided surgery should be used to counteract the drill's tendency to move toward the newly formed bone. It is also recommended that, in cases with incomplete bone regeneration and fallen roots, for example, in lower-molar teeth, guided surgery should be used to counteract the drill's tendency to move toward the newly formed bone [108].

It should be noted that high accuracy of implant placement is essential for ideal results, and guided surgery significantly increases accuracy, but unfortunately, this method is not yet used exclusively. Some dentists prefer to perform the implant by the free-hand method, which seems to be due to insufficient information to choose the right method for the implant. Since both free-hand and guided implant surgeries have their own advantages and disadvantages, it is recommended that dentists to have more extensive studies in this field to increase their knowledge and understanding of the use of these two methods and to use any technique to get the best results. Finally, it is recommended that guided surgery be preferred over the free-hand method when there is a possibility of error.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

This article is a review and does not contain any studies with human or animal performed by any of the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Biomaterials and Biological Parameters for Fixed-Prosthetic Implant-Supported Restorations: A Review Study

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Because of the increasing use of dental implants, dental practitioners should understand the treatment and nature of peri-implant diseases (PIDs). This disease is a serious problem of dentistry, regarding epidemiology and therapy. Due to the increase in the practice of implantology as well as the increased number of implants placed every year, the rate of PID has widely increased. Peri-implant mucositis and peri-implantitis, gingivitis, and periodontitis are common clinical manifestations of the disease. PIDs are caused by chronic inflammatory processes in the tissues around an intraoral implant, with increasing incidence, and have become a health concern. Bacterial infections are involved in the pathogenesis of these diseases. The imbalance between the host response and bacterial biofilm results in tissue destruction. New challenges lie in the prevention, treatment, and diagnosis of PIDs. The aim of this overview was to focus on the nature of the disease itself, useful diagnostic criteria, common responsible bacteria, and the prosthetic effects of fixed restorations on the health of the periodontium since recognizing the parameters involved in the development of periodontal and PIDs will play a crucial role in preventing the progression and minimizing the complications of these diseases having a fixed prosthesis.

1. Introduction

Loss of teeth or part of a tooth due to caries and periodontal disease is one of the undeniable problems in today's society. Failure to replace a lost tooth over time has caused the condition of the remaining teeth to change, the coordinated function of the teeth gradually changing and causing many problems in chewing, speaking, beauty, health, and dental hygiene [1]. With restorations, large areas of missing teeth or crowns can be replaced [2]. However, successful treatment of patients by a dentist for the placement of fixed prostheses requires a series of knowledge. Plaque accumulation in fixed restoration can endanger the life of the tooth, and the importance of this issue is clear from the fact that the prevalence of microbial plaque is very high and exists in all age groups and social and economic classes [3]. Also, the accumulation of microorganisms and the formation of microbial plaque can lead to gingival resorption and periodontal degeneration and marginal caries and, consequently, tooth sensitization, crown looseness, and ultimately lead to periodontal disease [4]. The link between periodontal and host microbes is usually benign, but when certain bacterial species overgrow in the subgingival spaces, they can cause periodontal inflammation and destruction with loss of tooth connection and bone loss. In periodontitis, the onset of the disease is associated with tissue colonization by these pathogenic species [5]. The next stage is bacterial invasion or invasion of periodontal tissues by their pathogenic products,

reactions of bacteria, and their materials with host cells, and this results in tissue destruction, directly or indirectly, causing periodontal destruction. Bacterial species must be able to colonize the subgingival area to produce virulence factors that can directly (by producing enzymes or toxins) or indirectly (by producing antigens and activators) lead to the onset of a destructive inflammatory reaction in individuals and periodontal tissue injury [6]. Plaque reduction, usually above and below the gingival margin, can be a major factor in preventing periodontal disease and caries [7]. Plaque formation in fixed restorations is very important, so the use of appropriate materials to prevent periodontal disease is effective [8]. Also, because implants are prone to biofilm-induced inflammatory diseases, it is necessary for the dentist to perform examinations using clinical indexes. Dental implants biological complications include plaque-related diseases, and the clinical manifestations are peri-implant mucositis and peri-implantitis, gingivitis, periodontitis [9], and non-plaque-related conditions near dental implants, such as mucosal hyperplasia, implant mucosal recession, lesions caused by trauma, and different nonspecific clinical diseases [10]. Diagnosis of such plaque-induced and non-plaque-related peri-implant diseases is associated with plaque-related active infection, for example, bleeding on probing, radiographic bone loss, exudate/suppuration, and increasing the depth of probing pocket. The prevalence rate of peri-implant mucositis and peri-implantitis has been reported to be nearly 43% (range 19%–65%) and approximately 22% (range: 1%–47%), respectively [11, 12].

Generally, bacterial virulence factors are challenging for the host for mounting a response for minimizing the harmful impacts of the bacteria or byproducts of them. These factors are toxins releasing by living bacteria and also cell wall constituents of the bacteria as well as cell contents releasing on cell death. Although there are many species that are considered indigenous for the oral cavity, some species are linked to caries and some certain other species, such as *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*, and *Porphyromonas gingivalis*, are involved in gingival inflammation and periodontitis [13, 14]. Nonetheless, the host reaction is a crucial factor in initiation/progression of the disease since all sites colonized via inflammation-associated bacteria certainly are not inflamed. Thus, the host response against a tooth/restoration or abutment/restoration interface should be realized and is more important compared to only measurement of the levels or presence of some bacterial species [15].

The aim of this review study was to gather conclusive information on diagnostic criteria for peri-implant diseases and the effect of fixed restorations and cementation on the health of the peri-implant tissues.

2. Prosthetic Factors

2.1. Type of Restoration. It is demonstrated that crowns that have intracrevicular margins, made of several traditional and new biomaterials, result in desirable microbial and gingival responses while supporting by either titanium implant abutments or natural teeth [15]; nonetheless, the zirconia crowns can exhibit the least marginal gingival inflammation [16] despite the fact that there have been no differences between porcelain and ceramic fused to metal restorations on periodontal health [17].

2.1.1. Porcelain-Fused-to-Metal (PFM). Today, one of the most popular and usable restorations is ceramic metal restoration, which combines the power and precision of metal casting with the beauty of porcelain. Porcelain-fused-to-metal (PFM) has long been used in clinical practice and is therefore considered the standard material for implant-supported restorations [18]. This type of infrastructure provides sufficient power and satisfactory aesthetic results. PFM crowns are considered the golden standard for repairing damaged teeth. A survey of 80 dentists found that 70% of them used PFM crowns to restore patients' posterior teeth. Also, in recent years, the demand for crowns that look natural has increased because ceramic materials have a high degree of beauty [19]. PFM crowns are more beautiful than previous metal coatings but have a shorter life and strength. It also has more strength but less beauty against ceramic coatings. PFM crowns last longer than all-ceramic veneers and less than metal veneers. Other advantages include high strength, beauty, and more acceptable cost than zirconia and all-ceramic coatings [20]. PFM crowns can also be used for front and posterior teeth, even bridges and implants. PFM is resistant to abrasions and cracks and is also recommended for people who grind or harden their teeth because PFM is more durable than other dental crowns [21].

2.1.2. Monolithic Zirconia. Zirconia is a white alloy used in the place of gray metal and is the basis of bridges and prosthetic dentures. Zirconia veneers are used to enhance the beauty of yellow, discolored, and incurable teeth with methods such as bleaching, and zirconia veneers are also used to treat congenital discoloration, cleft palate, broken teeth, and decayed teeth [22]. Zirconia veneer is also used as a bridge or veneer for molar teeth, anterior teeth, and as a treatment for the smile line. The monolithic zirconia crown has high flexural strength and shows high fracture resistance in the posterior region [23]. Because protective screw implants can also be made from integrated zirconium, evaluating the clinical results of this type of restoration is invaluable. The extraordinary strength of the integrated zirconia is due to its diamond-like structure [24]. This material does not need any other metal to increase strength and is resistant to abrasion and unbreakable. However, there are many types of zirconia ceramic systems available [25]. But only three of them are used in dentistry, which include glass-infiltrated zirconia-toughened, magnesium cation-doped partially stabilized zirconia (Mg-PSZ), and yttrium cation-doped tetragonal zirconia polycrystals (3Y-TZP) [26].

Zirconia-based restorations promise a great alternative to metal-ceramic restorations. Both fully sintered and semisintered zirconia products appear to be clinically acceptable. Adaptation of zirconia frameworks made with CAM/CAD technique is clinically acceptable. From the

point of view of resistance fracture, fixed restorations of the zirconia particle are capable of withstanding occlusal physiological forces in the posterior region [27]. Clinical evaluations have shown that the clinical success of single restorations and zirconia bridges is excellent. It is similar to metal-ceramic restoration in terms of tooth preparation and cementing. Recently, surface preparation using simultaneous abrasion particle borne air and composite resin containing MDP-10 seems to be suitable for zirconia bonding. Despite the excellent clinical results in short-term research, we still need more clinical and laboratory studies to have long-term information about subzirconia restorations [28].

2.2. Type of Cement for Cementation

2.2.1. No Cement (Screw-Type Restorations). Unlike cement teeth restorations, screw-type restorations use screws to hold the teeth in place, which has several key advantages, including the ease of managing screw-retained reconstructions in the event of technical or biological complications, as crowns can be easily removed from the implant for further treatment, cleaning, or preparation. And, when removing the crown, the teeth are not damaged. Screw-type restorations are at a higher level in terms of implant hygiene than cemented dental restorations [29]. Since the adhesive coatings which are attached to the implant are more likely to cause infection, inflammation, and even bone loss, and inflammation often occurs when some adhesive leaks around the gum line and causes allergies. Therefore, it seems that screw-retained restoration is more desirable in terms of the long-term stability of the implant as well as the tissues around the implant and the implant infrastructure [30, 31].

2.2.2. Temporary Cement. The decision to use temporary cement versus permanent cement is made based on the amount of retention required. In the case of implant-based prostheses, the desirability of recyclability, ease of removal and cleaning of cement from the abutment, and the amount of retention required, affects the choice of cement type [32]. The tensile strength of temporary cement must be such that they can withstand horizontal and vertical forces during operation, but they must also be weak enough to allow the prosthesis to be removed without damaging the abutment or implant [33]. When using cement, an important issue is the removal of cement residues, which have been reported to cause peri-implantitis when not removed properly [34]. At the time of delivery of the final implant prosthesis, the prosthesis is often cemented with temporary cement. The temporary cement must be fully solidified and provide a suitable and sufficient grip for the restoration to be able to hold the function properly [35]. The use of implant-based prostheses with cement retention is increased due to the similarity [36] of restoration methods for natural teeth, optimal occlusal adaptation, aesthetic enhancement, create disabled matching, ease of operation, low cost, disabled proper casting, less likely porcelain fracture, gradual loading, [37] reduced crystal bone resorption, and [38] role as a shock absorber. The biggest drawback of the cement retaining coating technique is the lack of a reliable average [39] for retention. Another problem is the possibility of problems due to the inability to remove excess cement from the implant margin, which causes severe periodontal disease in 80% of cases [40]. Materials mentioned as temporary cement in dental sources include type I zinc oxide eugenol cement (ZOE) or unmodified ZOE cement, zinc oxide without eugenol (eugenol-free ZnO (EF-ZnO)), and compound dimethacrylate. Today, zinc oxide compounds are the most widely used in this field and are generally divided into two types: zinc oxide eugenol and zinc oxide without eugenol [41, 42].

Provisional cements are characterized by high solubility and exhibit poor tensile strength [43], which are advantageous while completing a provisional recementing or cementation a prosthesis that is linked to peri-implantitis [44]. Retrievability of prosthesis is possible for provisional cements [45, 46]. Nonetheless, cement-retained implant-supported prostheses possibly lose retention while using provisional cement. Based on a systematic review by Ma and Fenton, 17.6% loss of retention of cement-retained implant-supported prostheses happened while using provisional cements [47]. Table 1 addresses the characteristics of EF-ZnO and ZOE.

2.2.3. Semipermanent Cements. Adequate retention is provided by semipermanent cements for resisting repeated decementation, and they allow for retrievability. Zinc phosphate (ZnP) and glass ionomer (GI) are regarded semipermanent cements while using with cement-retained implant-supported prostheses [43, 49]. Semipermanent cements decrease the risk of decementation than provisional cements. When a cement's tensile strength is between provisional and permanent cements, it can be categorized as a semipermanent cement. GI and ZnP provide retrievability while using with zirconia or titanium abutments [49, 50]. A systematic review by Wittneben and Bragger showed a decementation rate of 0% for cement-retained implant-supported prostheses than ZnP [51]. ZnP and GI characteristics are provided in Table 2.

(1) Glass Ionomer (GI). Glass ionomer cement has been used in dentistry for about thirty years. This cement is like silicate cement in appearance and workmanship and similar to polycarboxylate cement in terms of adhesion. The composition of glass ionomer cements is complex and diverse. Calcium fluor aluminosilicate is the main constituent of this cement [52]. The three main components of glass ionomer cement used in dentistry are silica (SiO_2), alumina (Al_2O_3), and calcium fluoride (CaF_2), which, when bonded together, it forms a suitable glass structure for cement formation. In glass ionomer cement, the basis of the hardening reaction and the formation of cement is based on the acid-base reaction [53]. The glass ionomer hardening reaction is a long-lasting comet reaction that lasts up to one month after material replacement [54]. In fact, it can be said that glass ionomer is an unusual material with

Table 1: Provisional cements [48].

Cement type	Characteristics	Pros	Cones
ZOE	(i) Bactericidal (ii) Soluble (iii) Radiopaque (iv) Poor bonding with titanium (v) Lowest tensile strength (vi) High pH (vii) Perfect marginal seal	(i) Marked decrease in periodontal pathogens (ii) Lack of residual cement, leading to low chance of peri-implantitis (iii) Easy detection of excess cement. (iv) Excess cement is easily removed (v) Appropriate for provisionalization (vi) Biocompatible (vii) Low level of bacterial microleakage	(i) Gap generation at the abutment - prosthesis interface (ii) Repeated decementations
Eugenol-free zinc oxide	(i) Organic acid alternatives for eugenol (ii) Solubility (iii) Poor tensile strength (iv) Poor inflammatory soft tissue factors	(i) Hypoallergenic (ii) Elimination of the adverse effects of eugenol on resin polymerization when permanent cementation is considered (iii) Poor risk of peri-implantitis	(i) Higher microleakage (ii) Poor retention

Table 2: Semipermanent cements [48].

Type of cement	Characteristics	Pros	Cons
ZnP	(i) Lower tensile strength compared to GI, but higher compared to EF-ZnO (ii) Low viscosity (iii) Lack of adhesion to prosthesis or titanium (iv) Highest elastic modulus (v) During setting, has high solubility (vi) Lowest level of creep (vii) Highest radiopacity than various luting agents (viii) Cost effective	(i) Decreased risk of decementation compared to provisional cements (ii) Easy flow for better mechanical retention (iii) High rigidity, making it appropriate for regions subjected to high occlusal forces (iv) Dimensionally stable, leading to no generation of stress on full ceramic prostheses (v) Excess cement can be easily detected easy removal of excess cement than GI cements and resin (vi) Inexpensive	(i) Inappropriate for short prostheses or abutments with elevated prosthetic abutment gap (ii) Insufficient marginal seal
GI	(i) Proper mechanical strength as well as bonding to base metal alloys (ii) Critical manipulation at setting time (iii) High material creep deformation over time (iv) High water absorption (v) Low elasticity modulus (vi) Radiopaque (vii) An increase in retention over time because of continued polymerization (viii) Cost effective	(i) Proper retention (ii) There are some brands (GC Fuji Temp LT) radiographically	(i) Insufficient moisture control at setting time leads to high microleakage (ii) Microcracks with extreme dryness at setting time (iii) No dimensional stability (inappropriate for full lithium disilicate ceramic crown) (iv) Inappropriate in regions facing high occlusal forces

excellent properties and is different from other materials. It is semitransparent like tooth porcelain and sticks to tooth tissue [55]. These materials adhere permanently to the enamel and dentin, which causes the seam between the material and the dental tissue to close almost completely, preventing the penetration of caries, thereby preventing secondary caries [56]. Recently, it has been reported that these substances have the property of releasing fluoride for a long time and can absorb fluoride when exposed to fluoride solution, thus inhibiting the progression of caries in adjacent tooth tissue [57]. In fact, glass ionomer cement has the approval and basic properties such as biocompatibility with dental pulp, the ability to chemically bond to enamel and dentin, and release fluoride, which can play an important role in inhibiting bacterial growth and caries progression [58]. The release of glass ionomer cement fluoride seems to be the most likely reason for the inhibitory effect on acid production. The availability of fluoride from glass ionomer cement is controlled by pH, which is controlled by salivary phosphate and proteins [59]. In general, glass ionomers are widely used in the restoration of dental structures in clinical dentistry.

(2) Zinc Phosphate (ZnP). Zinc phosphate cement was introduced in the year 1880 and has been used successfully in dentistry since then [60]. This cement is prepared by combining liquid phosphoric acid and powder containing zinc oxide and magnesium oxide. Although its use has declined with the introduction of other types of cement, this type of cement is still available in many countries [61]. Zinc phosphate cement has no chemical bond to dental structures and has medium compressive strength (62 to 101 MPa), low tensile strength (5 to 7 MPa), and high solubility (0.36%). After mixing, its pH is low, and about 2 and after 24 hours, it reaches 5.5 [62, 63]. Despite the low pH of this cement, it has been reported that it has no irritating effect on the pulp and it is biocompatible [64]. Due to the long history of using zinc phosphate cement, it is considered the golden standard of cement, and other types of cement are compared with it [63, 65]. Increasing the pH over time in zinc phosphate cement and decreasing fluoride release in glass ionomer cement can be the reason for the decrease in antibacterial properties. Also, an increase in pH in zinc phosphate cement occurs faster than a decrease in fluoride release in glass ionomer, and this may be the reason for the lower antibacterial activity of zinc phosphate cement than in glass ionomer [66, 67]. During a study, the antibacterial properties of seven types of cement (including RMGI, GI, composite resin, zinc phosphate, polycarboxylate zinc, ZOE, and zinc noneugenol oxide) against *Streptococcus mutans* were investigated, and it was observed that zinc phosphate cement showed the strongest antibacterial activity immediately after mixing compared to noneugenol, eugenol, and resin cement [68]. Due to a passive fit of implant-supported fixed partial dentures (ISFPDs), an essential gap is observed between the intaglio and abutment of the retainer for preventing a pressure on the supporting implants. For ZnP retention on natural teeth, a thin film thickness can be favorable; however, it is not significant for implant-supported abutments. Due to metal-to-metal marginal and internal gaps, ZnP solubility is a liability. This gap makes the cement exposed to oral fluids. When using ZnP as definite cement in multiple unit ISFPDs, ZnP cement dissolution may be seen. The cement dissolution of one or more retainers in a multiple unit ISFPD results in the transferring of the occlusal load toward the remaining retained units. Also, rotational force of the cement-retained implants may be observed; thus, the parafunctional and occlusal loads would be borne through the abutment-retained implants. Therefore, there is a risk for loss of integration, detrimental rotation, and overloading. ZnP cannot be indicated for ISFPDs or splints. Cement dissolution in single units may only involve recementation, when patients do not aspirate or swallow the crown [69].

3. Periodontal Factors

3.1. Peri-Implant Mucositis and Peri-Implantitis. An inflammatory lesion can be developed when a bacterial biofilm develops near a dental implant [70, 71]. Peri-implant diseases as infectious diseases are caused by developing a bacterial biofilm attached to the surface of implant that affects the implant supporting tissues and is very similar to periodontitis [72, 73]. However, the teeth and dental implants are different because teeth are characterized by the periodontal ligament including blood vessels as well as lymphatic channels. On the contrary, titanium dental implants are directly attached to the bone, which has been named as "osseointegration."

3.2. Risk Factors. Placing implants in the maxilla in male subjects and also cases who had a history of periodontitis is considered as risk indicators [74, 75]. There are many other risk factors linked to the development of PID, like smoking, poor oral hygiene and excess of cement. The occlusal overload role as a single risk factor has not well identified, in spite of the indications suggesting that overload alone is not linked to tissue destruction as well as PID without plaque. Occlusal overload possibly is involved when associated with preexisting inflammation or plaque accumulation. Nonetheless, the information is not clear [76, 77].

3.3. Etiology and Diagnosis. An imbalance between the host defense and the bacterial load causes peri-implant disease (PID) [9]. To prevent and appropriately handle complications, the proper diagnosis of peri-implant conditions (peri-implant mucositis, peri-implant health, and peri-implantitis) is of great importance [78]. Regular and routine examinations of cases with dental implants are used along with approved procedures for maintaining periodontal health. Detection of biological complications at a reversible and early stage can facilitate effective interception and prevent the progression of peri-implant complications [10].

Heitz-Mayfield defined the criteria for an accurate diagnosis of PID [79]. According to them, radiographic assessment and probing were proposed as the primary diagnostic tools.

A World Workshop on Periodontology (2018) reported bleeding on gentle probing as the main clinical characteristic of peri-implant mucositis, and it was stated that swelling, erythema, and/or suppuration may also be found. Peri-implantitis sites show the same clinical signs of inflammation, and also there is an increase in the recession and/or probing depth of the mucosal margin and also radiographic loss of bone compared to previous examinations. The following definitions were obtained from a review article by Renvert et al. [80, 81] and a consensus report by Berglundh et al. [82].

The following items are required for the diagnosis of peri-implant health:

(1) No clinical signs of inflammation

(2) No suppuration and/or bleeding on gentle probing

(3) The absence of an increase in probing depth compared to previous examinations

(4) No bone loss beyond crestal bone-level alterations caused by initial bone remodeling

Diagnosis of peri-implant mucositis requires the following:

(1) Presence of suppuration and/or bleeding on gentle probing with/without an increase in probing depth in comparison with previous examinations
(2) No bone loss beyond crestal bone-level alterations caused by primary bone remodeling

Diagnosis of peri-implantitis needs the following:

(1) Bleeding and/or suppuration on gentle probing
(2) An increase in probing depth in comparison with previous examinations
(3) Bone loss beyond crestal bone-level alterations caused by primary bone remodeling

3.4. Clinical Parameters

3.4.1. Bleeding on Probing (BOP). Insertion of the probe to the depth of the envelope will cause bleeding if the gums are inflamed and the epithelium of the envelope is atrophic or injured. Noninflammatory areas rarely bleed. In most cases, bleeding during probing, as a sign of inflammation, is detected earlier than gum discoloration [83]. However, non-bleeding discoloration may be observed during the probe [84]. Depending on the severity of the inflammation, the bleeding can vary from a thin red line around the gingival sulcus to severe bleeding [85]. After successful treatment, the bleeding stops during the probe [86]. The Gingival Bleeding Index is used to screen for gingivitis and periodontitis, determine the prevalence of the disease, evaluate treatment outcomes, and determine disease progression [87]. BOP, if performed as standard, is a reliable indicator of the health status of periodontal tissues [88]. To test for bleeding during probing, the probe is carefully inserted into the depth of the envelope and gently moved laterally along the edge of the envelope wall. Sometimes the gums bleed immediately after the probe comes out; in some other cases, bleeding occurs after a few seconds [89]. Therefore, the clinician should re-examine the bleeding 30 to 60 seconds after probe. As a single test, bleeding during the probe is not a good indicator of the progression of attachment loss. However, its absence is an excellent indicator for predicting the stability of periodontal status [86]. Bleeding during probing can be a good indicator of progressive attachment loss when bleeding has occurred in several areas of the progressive disease [90]. No BOP can indicate healthy soft tissue and a decreased chance of disease progression/development [91]. A diagnosis of peri-implant health could predict maintenance of implants over 20 years or more [92]. Peri-implant disease can be prevented by early detection of inflammation around implant. The BOP presence can suggest soft tissue inflammation and is used clinically for distinguishing between peri-implant mucositis or peri-implantitis and peri-implant health [11].

3.4.2. Clinical Attachment Loss (CAL). The CAL is one of the primary symptoms of periodontal disease. Attachment loss is the apical migration of the dentogingival junction—periodontal attachment apparatus—the result of an inflammatory response due to the formation of oral biofilm (Figure 1) [93]. The junction of teeth and gums consists of epithelial junctions and connective tissue junctions. The size of the junction of the teeth and gums is called the biological width, and the average is 2.04 mm [94]. Under healthy conditions, without loss of adhesion, connective tissue connectivity at the coronal side begins at the cementoenamel junction (CEJ), and epithelial connectivity is more coronal than connective tissue connectivity. In cases where there is a loss of adhesion, CEJ becomes apparent [95].

CAL measures the amount of adhesion loss that occurs by reference to CEJ. The extent of clinical adhesion loss is the distance between the CEJ and the depth at which gingival crevice probing is possible. When the gingival margin is on the anatomical crown, CAL is the result of subtracting the distance between the gingival margin and the CEJ from the probing depth. If these two values are equal, the CAL will be zero [96]. CAL is equal to the probing depth when the gingival margin is on the CEJ. When the gingival margin is at the apical CEJ, the CAL is greater than the probing depth, so in this case, the CAL (distance between the CEJ and the depth of the gingival groove with the possibility of probing) is equal to the sum of the gingival resorption values and the probing depth. Drawing the position of the gingival margin on the periodontal chart at the point where the probing depths are recorded helps to clarify this important point [97].

In many dental office management software, CAL is calculated automatically by adding the values of analysis and probing depth. This calculation is only accurate if both the depth of probing and the amount of analysis are entered correctly in the software [98]. When the amount of analysis is not entered into the software, many assume that the CEJ is flush with the gingival margin and assume that the amount of clinical adhesion loss is equal to the depth of probing. This calculation is not necessarily correct because many clinicians do not enter any value into the software when the CEJ is subclavian and invisible [99]. Thus, the automatically calculated CAL should be reviewed and criticized before being used to aid in diagnosis.

3.4.3. Probing Depth (PD). There are two different types of envelope depth, biological or histological depth, and clinical or probing depth. Biological depth is the distance between the gingival margin and the base of the gingival groove (the coronal end of the junctional epithelium) and can only be measured by very precise histological sections [100]. Probing depth is the distance between the gingival margin and the depth of the groove with the possibility of probing. The degree of penetration of the probe depends on the shape and size of the probe tip, the force used to insert the probe, the strength of the tissue, the convexity of the crown, and the degree of inflammation of the tissue [101]. The depth of probing is generally less than 3 mm in gingival health and greater than 3 mm in the presence of inflammation. In human periodontal pockets, the tip of the probe is inserted into the most coronal fibers of healthy and intact connective tissue attachments [102]. In a periodontal pouch, a probe

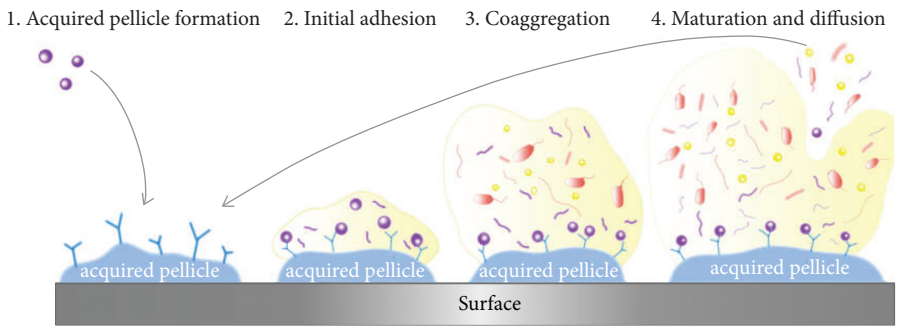

Figure 1: The formation stages of biofilm [177].

about 0.3 mm apically from the junctional epithelium penetrates into the connective tissue [103]. This is important in assessing the difference in probing depth before and after treatment, as reduced probe penetration is more likely to be due to a reduced inflammatory response, rather than attachment gain [104]. The depth of probing may change at different times, even in patients with untreated periodontal disease, due to changes in the position of the gingival margin. Therefore, the depth of probing may have nothing to do with connective tissue connections [105].

Probing depths around implants are associated with the soft tissue height around the implant as well as the primary placement of the implant. The pocket depth is associated with where and how deep the implant is located in the jawbone. Therefore, peri-implant tissue health exists surrounding implants with different levels of bone support. Probing depths is needed to be dependent on reference to the depths while placing the implants in the oral cavity after the healing process [106].

A force of 0.25 N is needed for probing to prevent damage to the peri-implant tissues and assess the presence of BOP, indicating the existence of inflammation in the peri-implant mucosa. It can predict no tissue support. PD should be examined regularly to detect BOP and suppuration and also to indicate an increase in depth, which is usually dependent on the supporting bone and loss of attachment [9]. Monitoring alterations in probing depth should be done for the diagnosis of peri-implant conditions. Increasing pocket probing depth indicates disease progression. The design of the prosthetic reconstruction may make probing at implants difficult. It is very important that the prosthetic reconstruction allows both easy access for oral hygiene measures and access for accurate probing around the implant. It should be noted that there is a major risk for underestimating the probing depths at implants when probing is performed with the prosthetic reconstruction in place [107].

3.4.4. Suppuration. Suppuration is caused by inflammation associated with neutrophil cell death in the soft tissues around the infected implants. Suppuration is a diagnostic factor for peri-implant disease [108], and its presence indicates the need for further evaluation and treatment.

3.4.5. Radiographic Evaluation. Taking a radiograph is recommended in the presence of clinical signs of disease (suppuration, BoP, and deepening pocket), for evaluating the presence of bone loss. For measuring bone loss at the site of implant, the bone height should be compared between visits and also when the implant healed in relation to baseline conditions. Bone loss around dental implants can be measured through radiographs. Long-cone parallel radiographic projection methods can evaluate crestal bone-level alterations [109].

Several variations in the extent of bone loss required for making a definition of peri-implantitis have been suggested [79]. An effective working threshold to diagnose peri-implantitis can be bone loss at implants 2 mm from the considered marginal bone level after early remodeling, which is called "biological bone remodeling" [109, 110]. When the implant type is known, the length of the implant thread pitch can be identified, which makes it possible to calibrate distances from the implant platform to bone-level contact (mm). In case of no previous radiographs, a bone-level distance of more than or equal to 3 mm apical to the most coronal part of the intraosseous part of the implant can indicate peri-implantitis. Therefore, it is essential to record radiographic and clinical data following prosthesis installation of implants for securing baseline criteria regarding the differential diagnosis of peri-implant diseases [109–111].

It is essential to take radiographs regularly for assessing the possible elevation in bone loss. Therefore, there is no settled, official classification to distinguish various levels of severity of PID, like the classification for periodontal disease; however, in 2012, Froum and Rosen proposed a classification [112] (Table 3).

For estimating advanced peri-implantitis defects in lingual, facial, and proximal bony lesions, cone beam computed tomography seems an accurate diagnostic tool [113]. In case of development of a radiographically radiolucent periapical lesion shortly following implant placement when the bone is osseointegrated in the coronal part of the implant, it is called retrograde peri-implantitis. A fistula can be developed in some cases [114, 115].

3.4.6. Gingival Recession (GR). The gums are part of the orthodontic canal that acts as a strong barrier against the penetration of stimuli into the periodontal tissue while

Table 3: Peri-implant disease classification.

Type	Characteristic
Early	PD greater or equal to 4 mm (suppuration and/or bleeding on probing) Bone loss of smaller than 25% of the length of implant
Moderate	PD greater or equal to 6 mm (suppuration and/or bleeding on probing) Bone loss of 25–50% of the length of implant
Advanced	PD greater or equal to 8 mm (suppuration and/or bleeding on probing) Bone loss of greater than 50% of the length of implant

covering the cervical region of the teeth and the alveolar appendages of the mandible and upper jaw [116, 117]. The normal position of the gums is 1–3 mm higher than the CEJ of the tooth, which moves to the CEJ over time [118, 119].

GR means moving of the edge of the gum from its normal position on the crown of the tooth to the lower levels relative to the CEJ [120]. Gingival resorption can be localized or diffuse and usually increases with age [121, 122]. GR occurs because of a combination of etiological and predisposing factors. Predisposing factors include keratinized gingival insufficiency, iatrogenic factors, bone fractures, and improper placement of the tooth in the dental arch [123]. Etiological factors include toothbrush damage, periodontal disease, ectopic frenum, and orthodontic treatment [124].

Some dental treatments can make the site more prone to gingival recession, such as preparation of dental incision under the gums, casting procedures in which the gums are removed, restorations, and veneers that are placed under the gums, and dentures that are not designed properly and the placement of the clasp, which increases the accumulation of plaque around the base tooth, causes GR [125]. However, one of the main causes of gingival recession is a gingival infection due to pathogenic bacteria after implantation, which can lead to implant destruction. When a gingival recession occurs, "envelopes" or gaps are created between the teeth and the gum line, making it easier to get the disease from existing bacteria. If left untreated, the supporting tissue and bone structures of the teeth can be severely damaged and can eventually lead to tooth loss [126].

3.5. Microbial Profile. The peri-implant biofilm bacterial composition was very similar to that of the neighboring teeth indicating that the microbial flora on natural teeth can be served as the biofilm generation reservoir around implants. Also, the biofilm microflora qualitative composition in peri-implantitis was very similar to that of periodontitis indicating why cases suffering from active periodontal disease are more prone to peri-implantitis. To support this theory, Kocar et al. [127] assessed cases with partially edentulous and fully edentulous patients and reported that the peri-implant as well as periodontal sulci of partially edentulous patients were not different regarding the microflora and share the same periodontopathogenic species; however, none of these bacteria were detected in the peri-implant sulci or the alveolar gingiva in patients with complete edentulous. Also, the existence of nonperiodontal microbial species, like *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus warneri*, and *Staphylococcus aureus*, has been reported around diseased implants [70, 128, 129].

3.5.1. Porphyromonas gingivalis (P. gingivalis). Today, more than 700 bacterial species can be identified in samples taken from the oral cavity [130] *P. gingivalis* is a Gram-negative, immobile, saccharolytic, rod-shaped, forced anaerobic bacterium associated with the onset and progression of periodontal degradation. This bacterium is found in small amounts in the mouth of healthy people [131], and it can be harmful when it attaches to the tooth surface and, by covering the tooth surface, leads to the formation of a mixed biofilm, which eventually leads to the spread of bacteria to the gingival fungus and forms the periodontal pocket [132]. Inside it is crevicular fluid, which is an inflammatory secretory source of nutrients essential for the growth of *P. gingivalis* [133]. The bacterium has a capsule that protects it against phagocytosis, also has fimbriae (for adhesion) and vesicles, and is able to produce a number of virulence factors [130]. *P. gingivalis* is the first pathogen of chronic periodontitis and the second most common pathogen in invasive periodontitis. One of the reasons why this bacterium has attracted the most attention is its high ability to escape the immune response. It is also able to alter the immune response of the host, thus creating a favorable environment for its persistence and other pathogens [134]. This bacterium causes the destruction of periodontal tissues by creating a number of virulence factors and extracellular proteases such as fimbria, lipopolysaccharide, gingipain, which is one of the main causes of periodontal diseases [130]. Virulence factors of *P. gingivalis* play an important role in the formation of biofilm and oral microbial dysbiosis. It is also detectable in 85% of subgingival plaques in patients with chronic periodontitis [135]. It is believed that this bacterium (even when it is in low abundance) is able to induce chronic periodontitis [136]. It has also been shown that *P. gingivalis* is able to interact with *Streptococcus gordonii* to form dental plaque and binds to GAPDH and surface proteins on the surface of *S. gordonii* to form *P. gingivalis* biofilms on the tooth surface [137]. LPS is one of the most important components in the wall structure of Gram-negative bacteria, including the periodontal pathogen *P. gingivalis* [138]. *P. gingivalis* LPS plays an important role in pathogenesis in periodontal tissues. The virulence properties of *P. gingivalis* LPS is determined by its lipid A component. The host cells respond to *P. gingivalis* LPS lipid A and cause inflammatory responses in the gingival tissues, thereby creating a favorable environment for pathogens that lead to the progression of periodontal disease [139]. Finally, it can be noted that *P. gingivalis* leads to inflammation and loss of alveolar bone in several ways, including upregulation of TLR2 expression and proinflammatory cytokine, NF-κB pathway, the mitogen-activated protein kinase (MAPK) pathway [130, 139].

3.5.2. Treponema denticola (T. denticola). *T. denticola* is a Gram-negative, motile, anaerobic bacterium associated with periodontal lesions. This bacterium is more common in patients with severe periodontitis than in patients with healthy periodontitis or gingivitis [140]. This microorganism produces proteolytic enzymes that can break down immunoglobulins (IgG, IgM, and IgA) and complement factors. The bacterium attaches to host cells and tissues, as well as matrix proteins and collagen-binding proteins and collagens that act as barriers to bone regeneration [141]. It can also bind to other bacteria, including *P. gingivalis* or other bacteria involved in the periodontal formation, which can lead to bipolar and nutritional interactions. The high motility of this bacterium and the presence of proteolytic enzymes and cytolytic agents in it contribute to the possible invasive ability of treponemes [142]. As periodontal treatment progresses, the depth of individual pockets and the amount of inflammation as well as the treponemal population decrease. *T. denticola* can cause periodontal disease in several ways: activation of IL, bone resorption, an inhibitor of superoxide production by leukocytes, enhancement of collagen phagocytosis by gingival fibroblasts, a monocyte-dependent suppressor for human lymphocyte response, suppression of fibroblast proliferation [143, 144]. Since the amount of *T. denticola* in plaque samples collected from deep pockets of patients with severe periodontitis is higher than healthy people or people with moderate disease, so it can be concluded that there is a positive and significant relationship between *T. denticola* and severe periodontitis [145, 146]. *T. denticola*, like *P. gingivalis*, is found mainly in the plaque below the gums and is found in periodontal pockets more than 4 mm deep. In a mouse study of periodontal disease, *T. denticola* infection was found to induce alveolar bone resorption [147, 148]. It was later shown that acylated dual and triple synthetic lipopeptides, which preferentially activate the TLR2/6 and TLR2/1-dependent pathways, play a key role in stimulating alveolar bone loss in the mouse model [149].

T. denticola is involved in the expression of several virulence factors. Among them can be the major surface protein (Msp), chymotrypsin-like protease (CTLP), and cell-associated lipooligosaccharide. These factors facilitate the integration of *T. denticola* within oral dental plaque biofilms as well as promoting its adherence and subsequent invasion into the host's cells. Although *T. denticola* lacks a specific adhesive structure such as fimbriae, it is able to adhere to various surfaces of the oral cavity, including teeth, host soft tissue, and microbes in plaque biofilms by various other factors, including Msp, lipooligosaccharide, and CTLP [150, 151].

This bacterium is able to penetrate the epithelial cell by disrupting tight cell connections. This penetration is facilitated by CTLP and the outer membrane. The immunomodulatory activity of *T. denticola*, which is facilitated by Msp and CTLP, is involved in its pathogenicity, including being able to elicit a strong antibody response in adult patients with invasive periodontitis [152]. It also inhibits the chemotoxic activity of neutrophils and suppresses the properties of human peripheral mononuclear cells and fibroblasts. It can also be said that *T. denticola* exerts cytopathic activity against epithelial cells and fibroblasts that represent periodontal tissue, in which these cytopathic activities lead to vacuolization, membrane damage, cell detachment, loss of tight intercellular contact, inhibition of proliferation, and cytoskeletal rearrangements [153].

3.5.3. Prevotella intermedia (P. Intermedia). *P. intermedia* is a short, immobile, Gram-negative, pathogenic anaerobic, rod-shaped bacterium with a rounded end. This bacterium has the ability to communicate with other oral bacteria. Among other things, it is able to communicate with Peptostreptococcus and lead to a dentoalveolar infection by increasing the virulence factor [154]. Various studies have shown that *P. intermedia* is associated with the incidence of acute necrotizing ulcerative gingivitis, pregnancy gingivitis, HIV-associated periodontal lesions, and periapical abscess [155]. In the oral cavity, *P. intermedia*, along with other pathogens, play an important role in the onset and subsequent development of polymicrobial periodontal disease. *P. intermedia* is visible in healthy and diseased periodontium, but its amount is higher in unhealthy periodontal than in healthy periodontal [156]. *P. intermedia* can be removed from odontogenic abscesses, saliva, the tongue, as well as subgingival sites of healthy and sick people [157]. According to a study by Maeda et al, the amount of *P. intermedia* in microbiological samples collected from the subgingival crevices or periodontal pocket was reported to be 71% in healthy individuals and 88% in patients with periodontal disease [158]. As we know, periodontal tissue destruction occurs mainly through cytokines and inflammatory proteinases released by immune cells in response to pathogens [159]. *P. intermedia* is able to destroy periodontal tissue through the expression of proinflammatory cytokines in human gingival epithelial cells and human periodontal ligament (hPDL) cells [160]. Also, MMPs produced by host cells, along with other proteinases, are responsible for the connective tissue destruction in periodontitis [161]. The results of a study by Zheng Wu et al. show that *P. intermedia* leads to the expression of MMP-9 in hPDL cells [162]. In this way, it can also cause damage and destruction of periodontal tissue. It is also able to directly separate ECM components of the periodontium by proteinases and endotoxins and indirectly destroys periodontal tissues through one or more of the five host-destroying pathways, including MMP pathway, osteoclastic bone resorption, phagocytic pathway, PMN-serine proteinase pathway, and plasminogen-dependent pathway [163]. Because the collagen degradation of unmineralized connective tissues and the organic component of bone in inflamed periodontal tissues is likely to be mediated by MMPs, *P. intermedia* plays an important role in the loss of connective tissue during progression by rapidly inducing MMP-9 expression [164].

4. Discussion

Replacement of missing teeth or part of a tooth that has been lost for various reasons has long been a problem in human

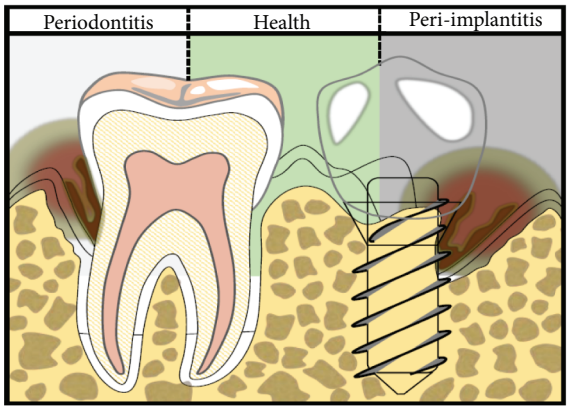

Figure 2: Periodontitis (left); peri-implant (right) [178].

societies that today can be compensated by using a variety of ceramic and metal castings or ceramic alone. But the successful implementation of such treatment requires compliance with a set of principles and methods of treatment [165]. And, the ultimate goal of the dentist is to perform proper restoration without damaging the healthy periodontal tissue [166, 167]. Since hygiene is one of the most important conditions for proper restoration and attracting positive feedback from patients [168], the use of a material that reduces plaque accumulation can be an important treatment to prevent periodontal disease (Figure 2) [169]. The increasing acceptance of dental implants can be explained by further development in both technical and biological aspects. At present, patients and dentists have a wide range of techniques and reinforcements, types of implants, and artificial structures to choose from. In this regard, it should be noted that decisions about the types and materials of superstructures can have a significant impact on the outcome of implant treatment. There are basically two types of infrastructure stabilization methods: cementation or screw retention. Both methods have been clinically and long-term use proved. However, the advantages and disadvantages of these methods are discussed among physicians. Periodontal examination and proper diagnosis are essential for intelligent treatment. The first step in periodontal diagnosis is to diagnose the disease. Probing around the implants is part of the examination and diagnosis [170, 171]. Then, the type, extent, and severity of the disease are determined with the help of various indexes, and at the end, it is time to identify the underlying pathological processes and their cause. Complete probing of periodontal pockets and measuring the depth of probing before, during and after treatment is essential for the diagnosis and monitoring of periodontal disease. In the present study, various clinical parameters such as BOP, PD, GR, and CAL were investigated. Probing is one of the most important and reliable parameters in the long-term examination of the soft tissue of the implant [171–174]. Prospective studies also emphasize that, like natural teeth, lack of blood during probing has a high negative predictive value and can be an indicator to predict the stability of the tissues around the implant. The clinical picture that distinguishes periodontitis from gingivitis is the presence of clinically detectable attachment loss in the periodontium. This condition is usually accompanied by the formation of a pocket and changes in the density and height of the adjacent alveolar bone. In some cases, gingival resorption may occur with attachment loss. Therefore, if PD is measured without CAL measurement, the progressive disease remains hidden and undiagnosed [175].

One of the risk factors for periodontal disease is mainly Gram-negative bacteria. Periodontal pathogens that have attracted the most attention of researchers in recent years include *P. gingivalis*, *T. denticola*, and *P. intermedia*. There is evidence that these bacteria are responsible for, or in some way related to, the attachment loss, especially in periodontitis. Evidence suggests that other microorganisms may also be involved in periodontitis. Although these pathogens are necessary for periodontitis, their presence is not sufficient for this purpose [176].

5. Conclusion

Today's knowledge states that maintaining soft tissue health is as important in the long-term maintenance and success of implants as osseointegration. Proper maintenance around the implant should be an important motivation for the dentist and the patient to prevent the disease, and this maintenance requires a series of knowledge about the causes of the disease. Usual maintenance of the tissues around the implant is vital but may be difficult for some patients. In this case, a reliable method in periodontal health should be used to prevent the effect of primary microbial accumulation on dental implants. In the present article, it is believed that, to minimize periodontal infections around fixed prosthetic restorations, physicians should be fully aware of the various microbial components and periodontal parameters [177, 178].

6. Future Direction

Although not new, dental implant procedure is considered a novel treatment in restoring missed teeth. As a progressing field in dentistry, more studies are required for establishing standards regarding peri-implant diseases, correct diagnosis, coherent and evidence-based approach toward treatment of these disease, recognition of the differences between implant restorations and natural teeth, prosthetic driven implant surgery for achieving the most natural design of the lost tooth, and finally developing more biocompatible cements to preserve the tissue and to prevent any peri-implant diseases in cases of cement excess. These are all factors for respecting oral biology, the only one defensive barrier that does not allow us to invade the oral natural structure.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

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The authors would like to thank the colleagues.

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