

Comparative Effects of Bioglass and Zinc-Doped Bioglass on VEGF and FLT1 Gene Expression in Wound Healing

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Abstract

Tissue engineering improvements have allowed for the creation of scaffolds to support wound healing by providing cells with surfaces for attachment, migration, and proliferation. Research on the use of bioactive materials such as bioglass and zinc in collagen-based scaffolds has shown promise in enhancing tissue regeneration. In this study, the functional activity of BG, ZBG-integrated polyurethane (PU), polycaprolactone (PCL), collagen, and BG scaffolds was evaluated on wound healing.

Results indicated that scaffolds containing bioactive glass with added zinc exhibited a significantly higher wound healing effect through higher VEGF and FLT1 expression compared with the control. The combination of PU and PCL with collagen can support cell adhesion, proliferation, and regeneration of the extracellular matrix. The study investigates the translational potential of PU-PCL/collagen bioglass-based scaffolds, specifically the role of zinc-enhanced scaffolds, in accelerating the wound healing process through promoting angiogenesis and cellular responses associated with tissue regeneration. The results justify the pre-clinical application of multilayer scaffold in advanced wound management and offer a promising strategy for enhanced healing in skin-injured patients.

1. Introduction

The skin, being the largest organ of the body, acts as a protective barrier between the external elements and the internal systems, providing protection from pathogens and environmental stressors. Wound healing is a complicated biological process that depends on factors like the extent of wound injury, its type, blood circulation, nutritional status, and immunity of the individual. Newer developments in tissue engineering accelerate this process; bio-substitutes and biomaterials accelerate the inherent mechanism of the body [1]. One of the most critical aspects of tissue engineering involves the preparation of three-

dimensional scaffolds, while the unique hierarchical architecture of skin tissues is relevant to efficient cell and tissue culturing. By providing such scaffolds, the cells of connective tissues can adhere and migrate, proliferate, and differentiate into functional tissue. The utilization of collagen in the majority of scaffolds is due to its appropriate mechanical properties and Olives, a cornerstone of the Mediterranean diet, are valued for their nutritional profile and unique microbiota, harboring potential probiotic contenders like *Lactobacillus plantarum* and *L. pentosus* [as live microorganisms that confer health benefits upon ingestion in adequate amounts, primarily].

cell-adhesive functional groups, which can facilitate cell adhesion and proliferation. Specifically, electrospun nanoscale collagen fibers have appeared to enhance the attachment and growth of fibroblasts significantly [2]. cell-adhesive functional groups, which can facilitate cell adhesion and proliferation. Specifically, electrospun nanoscale collagen fibers have appeared to enhance the attachment and growth of fibroblasts significantly [3]. For more extensive skin damage, collagen is often combined with bioactive materials such as silicate bioactive glass (BG). Among various bioactive materials, 45S5 bioglass is particularly effective in promoting fibroblast activity and angiogenesis. It forms bonds with living tissues, promoting regeneration by enhancing cell adhesion, migration, and proliferation [4]. Incorporating zinc ions into bioglass (ZBG) adds antibacterial properties and enhances keratinocyte migration and re-epithelialization, both critical for wound healing [5]. Zinc also activates key signaling pathways like MAPK/ERK and Akt, VEGF which are involved in cell proliferation and migration and angiogenesis[6,7]. A major problem in wound healing is the stable release of growth factors and ions. In this respect, a multilayered scaffold has been designed to provide temporal release of bioactive molecules. These scaffolds can promote neovascularization: the formation of new blood vessels-a critical event in wound healing. VEGF regulates angiogenesis and mainly enhances the formation of new blood vessels to ensure blood supply and delivery of nutrients at the wound site. VEGF also increases vascular permeability, which facilitates the passage of nutrition and other important cells to the injured tissue [8]. Bioglass has been found to induce the expression of VEGF, thereby enhancing angiogenesis through the release of bioactive ions that subsequently activate cellular responses and support wound healing [9]. Similarly, the FLT1 gene encodes a receptor involved in controlling angiogenesis through the binding of VEGF to activate the signaling pathway that enhances the proliferation and migration of endothelial cells. These are essential processes where oxygen and nutrients need to be delivered to the healing tissue. FLT1 also takes part in the recruitment of fibroblasts and inflammatory cells, essential in tissue repair, by modulating the degree of inflammation to ensure a sped-up course of healing. Bioactive glass has been demonstrated to express FLT1, encouraging the formation of new blood vessels by facilitating the release of bioactive ions that enhance the activity of endothelial cells [10]. Polyurethane (PU) is a very versatile polymer widely used in tissue engineer-

ing due to its tunable mechanical properties along with biocompatibility and biodegradability. It often finds application in the fabrication of scaffolds that enhance cell proliferation, adhesion, and tissue regeneration. These scaffolds should degrade with time in the body at a rate that would help in tissue repair and remodeling. In most cases, PU is combined with other biomaterials or bioactive compounds to enhance its effectiveness in many areas, such as wound healing. The strength and elasticity of the material make it especially suitable for scaffolds in dynamic tissues which are able to bear mechanical stress: heart valves, blood vessels, and cartilage [11]. Polycaprolactone (PCL) is a biodegradable, biocompatible polymer that is commonly used in tissue engineering due to its excellent elasticity, mechanical properties, and extremely slow degradation rate. Its low melting point allows for easy processing, while the material can be presented in various forms, including films, fibers, or scaffolds. The applications of PCL in tissue engineering span further, from bone, cartilage, skin, to vascular tissue regeneration. Its properties support cell attachment, proliferation, and differentiation; it would be ideal for application in wound healing, bone regeneration, and drug delivery systems. The versatility of PCL and compatibility with other biomaterials, such as collagen, enhances its effectiveness to create scaffolds that mimic the extracellular matrix, thus providing an appropriate environment for tissue regeneration [12]. This work tries to investigate an inductive effect of BG and ZBG on the level of expression of VEGF and FLT1 genes involved in the process of wound healing. Moreover, it investigates the efficiency of polyurethane, polycaprolactone, collagen type II, and bioactive glass multilayer scaffolds in the promotion of wound healing. The research will explain in detail the role of these scaffolds in tissue regeneration and their potential to promote wound healing with the use of advanced therapies for wound care [12].

2. Materials and Methods

2.1. Synthesis of Zinc Ione doped Bioglass

The bioglass, BG, and zinc-doped bioglass, Zn/BG, were prepared using SiO₂, Na₂O, CaO, and P₂O₅ by the sol-gel method described in earlier works [13]. In summary, 0.064M (13.33 g) TEOS was mixed with 0.1M HNO₃ catalyst (30 mL); then, under continuous stirring, hydrolysis was carried out for 30 minutes at ambient temperature.

Within 45 min, TEP, calcium nitrate tetrahydrate, and zinc nitrate hexahydrate were added to the mixture and further stirred for one more hour at room temperature until hydrolysis was complete. With the purpose of incorporating zinc oxide in the bioglass structure, 5 mol% zinc nitrate hexahydrate was added. The obtained sol was aged for ten days in a tightly closed Teflon container with the purpose of allowing gelation and condensation processes. The gel was subsequently dried at 70°C for three days and further dried at 120°C for two days. The dry gel was filtered by a 90- μ m mesh filter (170 meshes) and then heated at 700°C for 24 hr to stabilize the glass structure. All chemical reagents were obtained from Merck Company, Germany.

2.2. Synthesis of scaffolds

The details of the fabrication method for the electrospun three-layer scaffold, used in this work, were described previously in our earlier project(14). Shortly, the first layer was prepared from polyurethane (thermoplastic PU, Desmopan), dissolved at a concentration of 7% in a solvent mixture of tetrahydrofuran (THF, Merck) and dimethylformamide (DMF, Merck) in the ratio of 75:25. The second layer was prepared with polycaprolactone (PCL, 70 kDa, Sigma) and type II collagen (Medzist), adding either 45S5 bioglass or zinc-doped bioglass. The last layer was prepared from a mixture of PCL and collagen without nanoparticles. Fabrication: The fabrication of this scaffold was done using an electrospinning machine (Nanoazma Company, Iran). The solutions were electrospun successively: PU, PCL-collagen-bioactive glasses, and PCL-collagen, thus giving a very characteristic three-layer scaffold. In this process, each layer was electrospun for 2.5 hours while changing parameters of voltage, flow rate, collector speed, and distance from nozzle to collector. For collagen cross-linking, 1-Ethyl-3-(dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) were used, both provided by Merck.

2.3. FTIR assay

FTIR assay the chemical groups of all polymers and the peaks of the nanoparticles were assessed using Fourier-Transform Infra-Red (FTIR, Thermo Nicolet model: NEXUS 670, USA) spectroscopy. The background of the FTIR spectrum was normalized using the KBr pellet method for all spectra, and the wavelength region was recorded between 500 and 4500 cm^{-1} . The scanning speed was 120 mV/min , and the resolution

was 4 cm^{-1} .

2.4. X-ray diffraction (XRD) analysis

To investigate the crystal structure of Zinc ion-doped bioglass used in the scaffold, the powder sample is subjected to X-ray diffraction analysis using an XRD device with wavelength $\lambda = 1.54 \text{ \AA}$. The dwell time in each step is one second, the step size is 0.058 degrees, and the range of 2θ is chosen between 10 and 80 degrees. The results will be compared in both control and control groups.

2.5. Animal wound model making

Animal studies were performed on 6 adult male Wistar rats weighing 200 to 220 grams, obtained from the Laboratory Animal Sciences Center of Baqiyatullah University in Tehran. All animal studies were conducted according to the regulations approved by the Islamic Azad University of Parand, and this project was allowed to perform the animal phase based on the contents of the contract with the ethical code IR.IAU.PIAU REC.1399.003. All rats were kept under standard conditions with free access to food and water. The animals were first anesthetized with ketamine (80 mg/kg , Sigma) and xylazine (6 mg/kg , Sigma), and then, the hair on the animal's back was shaved to remove the hair completely. Then, the wound area is disinfected, and using an 8 mm punch, we make a full-thickness wound on the back of the animal. Four wounds are made on the back of each mouse for all four groups which are No treatment group, PU-PCL/Collagen, PU-PCL/Collagen/BG, PU-PCL/Collagen/ZBG. Mice were observed for two weeks, and histological analysis and gene expression evaluations were performed.

2.6. Histological analysis

Tissue samples harvested on days 7 and 14 were prepared for histological investigations. Harvested tissues were fixed in 4% paraformaldehyde (Sigma) for 24 hours and then embedded in paraffin (ThermoFisher) after processing. Paraffin-embedded samples were cut into 6 μm thick sections with a microtome (Leica 2135, Germany) and dehydrated through a series of ethanol dilutions. A Masson's trichrome method was used for staining the prepared sections, which were viewed under a light microscope.

2.7. Gene expression essay

In the present procedure, at days 3 and 7 after surgery, wound tissue was taken from all the animals at the four wound sites and the samples were immersed immediately in ice-cold PBS. Using Trizol reagent (Sigma), the tissues were broken up in liquid nitrogen and total RNA was prepared. RNA contamination by protein was detected by dilution of 2 μ l extracted RNA in 98 μ l PBS. The OD was read using the NanoDrop, Thermo Fisher Scientific, at wavelengths 260 and 280 nm. Synthesis of cDNA was done using a kit bought from Sigma. RT-PCR: The genes were tested using the Rotor-Gene Q Corbett software, with all reactions done in triplicate. The threshold cycle was obtained in an Excel file after applying the Mellat program to all samples upon completion of the PCR reactions. Rotor-Gene Q (Corbett) software was used for data analysis. The specific primer pairs for the VEGF and FLT1 genes are shown in Table 1. Target gene expression was normalized against the GAPDH gene as an endogenous reference by the $2^{-\Delta\Delta Ct}$ method, and all groups' values were calibrated by taking TCPS as a reference.

Table 1. Primers used in Real Time PCR essay

Oligo name	Seq(5-3)
Flt1-F	CGGCAGACCAATACAATCCT
Flt1-R	TGTATTGAGGTCCGTGGTGA
VEGF-F	GCAATGATGAAGCCCTGGAG
VEGF-R	CCTATGTGCTGGCTTTGGTG
GAPDH	CACCACACTGAATCTCCCCT
GAPDH	TGGTTGAGCACAGGGTACTT

2.8. Statistical analysis

The collected data were analyzed using SigmaPlot software, with all experiments performed in triplicate, except for the FTIR spectroscopy analysis. The results are presented as the mean $\pm$ standard deviation (SD). A Student's t-test was applied to assess differences between the control and experimental groups, with statistical significance set at a p-value of ≤ 0.05 .

Results

3.1 FTIR analysis

The chemical groups of PU, PCL, and collagen, as well as the nanoparticles, were identified by FTIR. The results have been previously reported (14). FTIR identi-

fied chemical groups in polyurethane (PU), polycaprolactone (PCL), collagen, and nanoparticles in both cross-linked and non-cross-linked scaffolds. Peaks for PU, hydroxyl, and carbonyl groups were observed, along with CH₂ and CH stretching peaks. Collagen's amide peaks remained unchanged after cross-linking, confirming its structural integrity. Bioglass nanoparticles showed Si-O-Si, carbonate, phosphate, and calcite peaks, with some shifts due to polymer interactions. Zinc ions caused minor shifts, but metal doping had no significant effect on the spectra.

3.2 XRD

The crystal structure of bioglass nanocomposites is of considerable importance and significantly impacts their structure and biological behavior. With the help of the XRD technique, it is possible to evaluate the crystal structure of biological nano-glasses. It can be seen in Figure 1 that the pure sample S1 has two crystal structures at angles of 21.4 and 23.73 degrees. In sample S2, where BG nanoparticles are placed, the amount of crystalline plates has increased dramatically, possibly due to the better interaction of this nanoparticle with the polymers used. The crystalline structures are significantly reduced in sample S3, where zinc nanoparticles are located. It seems that in this sample, these nanoparticles have been able to reduce the regular parts in this sample by penetrating the crystalline structures and making the sample move toward becoming amorphous.

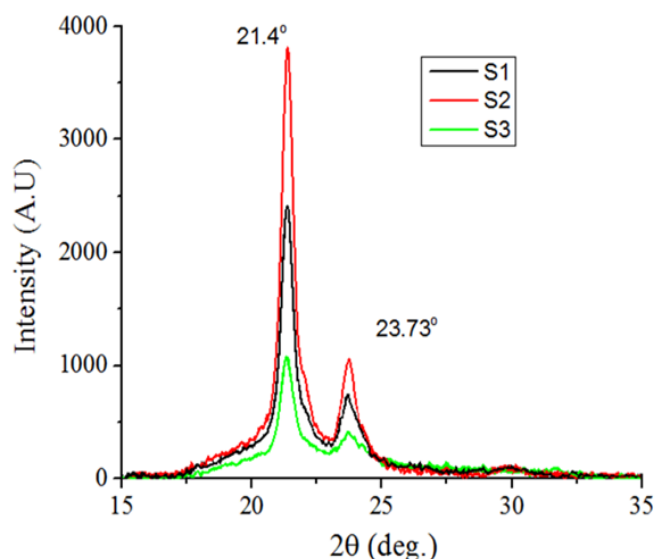


Figure 1- XRD analysis. S1 control group scaffold, S2 scaffold with BG, S3 scaffold with ZBG

3.3 Macroscopic analysis wound healing

The effect of fabricated scaffolds on wound closure in rats was investigated. This evaluated was done on days 7 and 14. Untreated wounds were considered as a control group. Wounds covered with PU-PCL/Collagen, PU-PCL/Collagen/BG, and PU-PCL/Collagen/ZBG scaffold. different effects on wound healing at time points showed different through macroscopic analysis. Wounds treated with scaffolds containing zinc nanoparticles, as shown in Figure 2, showed wound healing much faster than other groups in the early days. After 14 days, they were provided complete wound closure with a value of $98.5 \pm 3\%$.

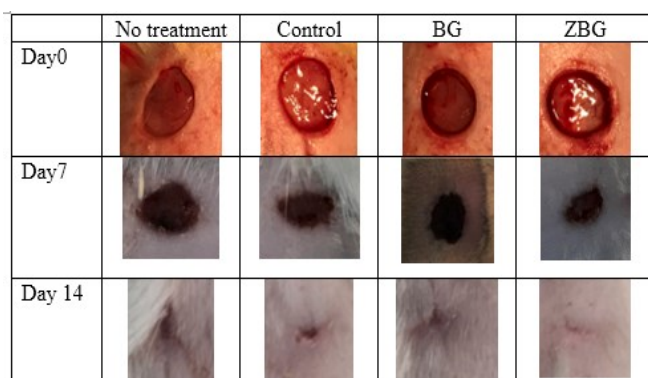
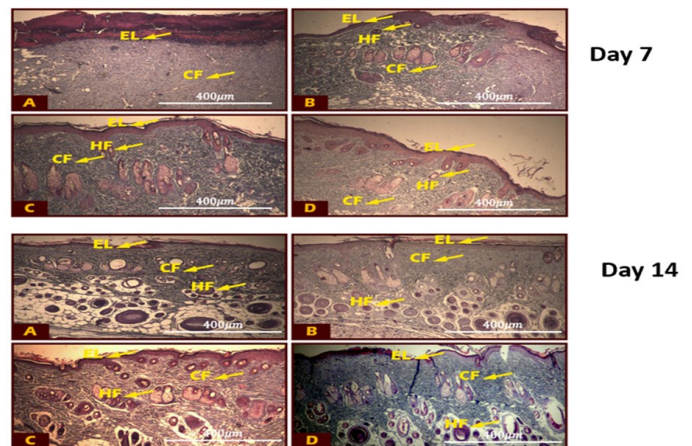


Figure 2- An overview of the wound healing process during 14 days of treatment

3.4 Microscopic analysis of wound healing

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Figure 3- Micrographs of Masson's trichrome and skin



wounds on days seven and fourteen. A) group without treatment. B) group treated with control scaffold. C) BG scaffold-treated group. D) ZBG scaffold treated group. Abbreviations: epidermal layer: EL, collagen fibers: CF, hair follicle: HF.

3.5 Gene expression analysis

The expression of genes involved in wound healing was evaluated by RT-PCR (Figure 4). The obtained data showed that three days after the operation, the scaffolds of the control group led to significantly higher levels of VEGF and Flt1 compared to the scaffolds of the control group ($P < 0.05$). However, increased expression was observed in the scaffold of the control group compared to the experimental groups. On the seventh day after surgery, an increase in VEGF expression was observed in the control group compared to the experimental groups. Furthermore, as seen in Figure 4, all synthesized scaffolds induced VEGF and Flt1 expression on day seven after surgery.

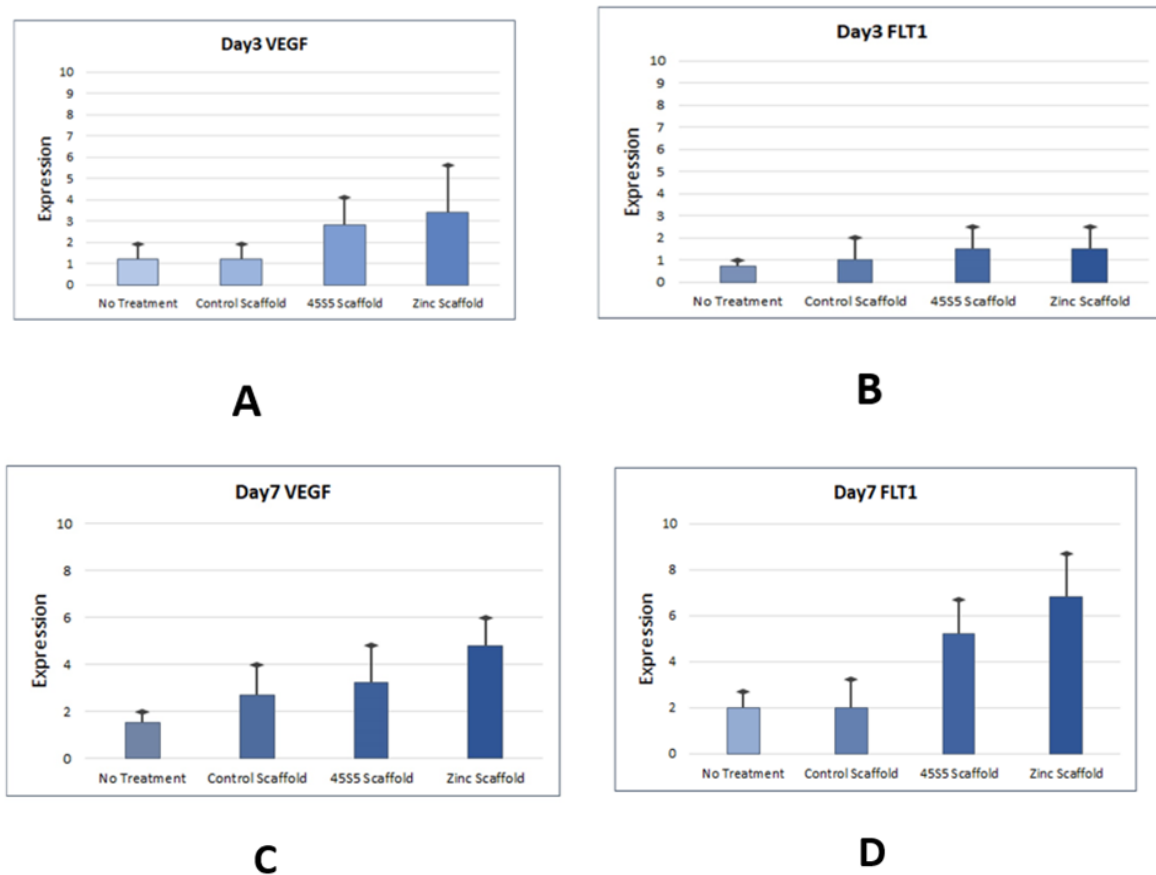


Figure 4 - Expression level of VEGF and FLT1 genes. A,B three days after surgery, C,D seven days after surgery.

Discussion

The design of the dressings that affect wound healing is an optimal biocompatible skin substitute that guides cell adhesion to facilitate infiltration, proliferation of fibroblasts and keratinocytes, and angiogenic potential for wound healing. Wound healing in wounds covered by bioactive glass scaffolds was higher than in the control group. The results show that using bioactive glass in the composition of scaffolds increases the speed of wound healing and the expression of VEGF. Many studies have shown that using PU polymer in the scaffold structure leads to higher surface tension in the scaffold (15). Also, according to the results from the studies, scaffolds containing PCL polymer have shown potential for tissue engineering applications due to their mechanical and morphological properties (16). Many studies have shown that combining artificial polymers with collagen provides an ideal system for tissue engineering applications (17). Due to collagen's increased biocompatibility and biofunctional roles, they can promote cell adhesion, maintain cell differentiation, di-

rect cell growth, and support ECM function (18). Collagen is the main component of connective tissues and is characterized by the presence of a proline-rich peptide-binding domain. Type I, II, and III collagens are the most common collagens and are called classic fibril forming collagens. Collagen has a relatively stable structure due to the formation of covalent bonds among fibers. However, its proteinaceous nature determines its biodegradability [40]. Collagen is widely used as a significant component in wound dressings due to its hemostatic and proliferative effects in fibroblasts and keratinocytes (19). Collagen is electrospun with other natural and artificial polymers and cross-links to form bonds with various materials. The results of the studies have shown that the combination of two natural and synthetic polymers, PCL and collagen nanofibers, did not show any evidence of cytotoxicity and increased cell adhesion and proliferation in natural human fibroblasts. The present study's results indicate collagen's influential role in ECM regeneration and neovascularization(20).

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Bioactive glasses (BG), such as 45S5 Bioglass, composed of 45% SiO₂, 24.5% Na₂O, 24.5% CaO, and 6% P₂O₅ (21) are the first synthetic mineral materials reported to be able to accelerate wound healing (22). The bioactive glass composite scaffolds in this study have acceptable biocompatibility for cell growth. Previous studies have shown that bioactive glasses can stimulate the proliferation and migration of endothelial cells and fibroblasts to wound sites, increase collagen deposition and the formation of new blood vessels, anti-inflammation and ultimately accelerate the wound healing process (23). In addition, bioactive glass not only stimulates the expression of essential growth factors in cells but also enhances cell-cell communication between endothelial cells or between endothelial cells and fibroblasts(24,25). Hoppe and colleagues have shown that various ions released through the dissolution of bio-glasses in biological solutions produce antibacterial, anti-inflammatory, and angiogenic effects (26).

Cordero and colleagues showed that the scaffold containing 45S5 biological bioglass as well as Zn can observe the antibacterial effects of Zn against E. coli (27). Because the growth of fibroblasts is necessary for skin repair, this study's beneficial result shows better skin regeneration due to the presence of zinc ions. The mechanism of this method includes Zn²⁺ ions reaching the wound area and then fibroblast proliferation (28). The higher expression of FLt1 and VEGF with the fabricated scaffold in the presence of bioactive ZBG indicates the vital role of these ions in stimulating the dermal molecular signaling pathway(29). This result is consistent with previous studies in which the level of VEGF expression was investigated on the same scaffold (30,31). In this study, the amount of collagen fibers, followed by re-epithelialization, has increased in the scaffolds containing bioglass, consistent with the results reported in other studies conducted on a full thickness mouse wound model.

Conclusion

In conclusion, this study highlights the potential of polyurethane (PU), polycaprolactone (PCL), collagen,

and bioactive glass (BG) scaffolds, particularly zinc-enhanced bioglass (ZBG), in promoting wound healing. The scaffolds demonstrated enhanced biocompatibility, mechanical properties, and ability to induce cellular responses necessary for tissue regeneration. The combination of synthetic polymers like PU and PCL with natural biomaterials such as collagen creates an environment that supports cell adhesion, proliferation, and the release of bioactive molecules. The results of the study confirmed the significant role of bioactive glass in wound healing, particularly through the induction of VEGF and FLT1 gene expression. These genes are critical for angiogenesis, which facilitates the formation of new blood vessels, a process vital for the delivery of nutrients and oxygen to the wound site. The incorporation of zinc ions into the scaffolds further contributed to the antibacterial properties and promoted fibroblast activity, accelerating the wound healing process. The study underscores the importance of multilayered scaffold designs that allow the temporal release of bioactive molecules, such as ions and growth factors, to ensure a controlled and sustained healing response. The findings pave the way for further development of advanced wound care therapies, combining natural and synthetic biomaterials for efficient tissue regeneration and accelerated wound healing.

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