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Abstract

Advances in tissue engineering have enabled the development of engineered bone constructs, which offer a promising alternative to traditional autografts and allografts for repairing bone defects for both internal and external transplants. The purpose of this study is to compare the effects of hydroxyapatite nanocomposite scaffold and titanium dioxide/hydroxyapatite nanocomposite scaffold on radius bone defects. PLA-gP (HEM-co-NIPAAm) hydrogel is used as highly effective supports for nanomaterials due to their combination of tunable porosity, mechanical flexibility, and biocompatibility. In the present study, nine adult male New Zealand white rabbits were selected to undergo radius bone surgery on both hands. These rabbits were randomly divided into three distinct groups (n=6 per group) for further examinations: 1) control group, no implant (comprised of six radius specimens), 2) PLA-gP (HEM-co-NIPAAm)/Hydroxyapatite nanocomposite implant (consisting of six radius specimens), and PLA-gP (HEM-co-NIPAAm)/TiO₂/Hydroxyapatite nanocomposite implant (consisting of six radius samples). The nanocomposites were synthesized and characterized by (XRD), Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), Scanning electron microscopy-energy dispersive X-ray spectroscopy (SEM/EDX) to investigate their physicochemical properties, and the cytotoxicity was investigated by MTT assay. The nanocomposites are prepared in cylinder shape corresponding to the defect size. The as prepared cylinder was implanted to fill the defect in groups 2 and 3, while the control group was left untreated. A unilateral 10 mm critical-size defect was created in the mid-diaphyseal region of the right radius under general anesthesia. Animals were monitored for 5 weeks, and bone regeneration was evaluated using serial radiographs X-ray scans, micro-CT, histopathology, and histomorphometry. The findings revealed that the group PLA-gP (HEM-co-NIPAAm)/TiO₂/Hydroxyapatite nanocomposite implants displayed superior ossification when

compared to the PLA-gP (HEM-co-NIPAAM)/Hydroxyapatite and control groups. Consequently, incorporation of TiO_2 with hydroxyapatite nanoparticles represents a viable choice for the rectification of bone imperfections in rabbits.

Keywords: TiO_2 NPs, PLA-gP (HEM-co-NIPAAM) copolymer, Hydroxyapatite, Radius, Bone healing

1. Introduction

The importance of bone scaffolds has increased significantly due to the increase in surgical procedures and advances in the treatment of skeletal abnormalities in humans and animals. These scaffolds are particularly important in repairing complex fractures, removing bone tumors, correcting congenital skeletal deformities, accelerating the healing process, spinal fusion surgeries, joint replacements, and dental procedures [1]. In the past, various methods were used to replace lost bone, with bone grafting (autograft) being the most effective and widely used [2]. However, advances in tissue engineering have now made it possible to produce bone scaffolds in the form of nanoparticles. Nanomaterials include a wide range of particles such as nanofillers, nanocrystals, nanotubes, nanofibers, nanowires, and nanofilms. Depending on specific requirements of the application, different types of synthetic nanoparticles can be produced using different techniques [3]. Natural bone is composed of collagen protein fibers and apatite nanocrystals, which form a nanocomposite. This combination makes it suitable for mimicking the extracellular matrix (ECM) in bone tissue regeneration [4]. The ECM is composed of both organic and inorganic phases. The inorganic phase contains crystalline hydroxyapatite nanoparticles with the formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, while the organic phase is composed of type I collagen and varying amounts of glycosaminoglycans, proteoglycans, and glycoproteins [5, 6]. Incorporating hydroxyapatite into composites can greatly increase bone conductivity because it constitutes a major part of the bone composition. Furthermore, hydroxyapatite nanoparticles have been found to enhance the growth and adhesion of mesenchymal stem cells, increase alkaline phosphatase activity, and facilitate the precipitation of calcium ions. This is due to their larger surface area and increased reactivity [7]. However, the brittleness of hydroxyapatite has limited its practical application. To address this problem, researchers have explored combining it with various biodegradable materials such as collagen,

gelatin, chitosan, and starch to create composites. In recent years, research efforts have increasingly focused on the development of bone substitute materials with optimized biological and mechanical properties, particularly through the incorporation of inorganic nanoparticles into polymer matrices [8, 9, 10]. About half of these efforts involve creating composites that mimic the structure of real bone. This approach involves combining organic materials with mineral fillers that stimulate the growth of mineral crystals in a way that mimics natural bone growth [10]. A polymer known as polylactic acid (PLA) has attracted much attention from researchers in the field of bone tissue engineering. PLA is attractive because it is biocompatible, biodegradable, and easy to prepare. It can be degraded by body fluids and broken down into natural materials [10, 12]. Hydrogels function as highly versatile supports for nanomaterials by stabilizing particles against aggregation, improving biocompatibility, modulating diffusion and release profiles, and enabling synergistic stimuli-responsive properties through their tunable, water-rich polymer networks. Among nanomaterials, titanium dioxide (TiO_2) is widely used in bone regeneration materials due to its excellent mechanical stability, biocompatibility, and intrinsic antibacterial activity, which plays a critical role in preventing implant-associated infections during bone healing [13]. Studies conducted in laboratories and clinical settings have demonstrated that TiO_2 nanoparticles can induce cell movement, depending on the concentration and size of the nanoparticles. These nanoparticles have been investigated for their potential in bone regeneration with promising outcomes [14]. TiO_2 nanoparticles can activate macrophages, leading to cell migration and the repair and regeneration of damaged tissue [15]. The photocatalytic reactions occurring on the surface of TiO_2 nanostructures play a crucial role in cellular responses, making a high surface-to-volume ratio essential for achieving desired outcomes [16]. A previous study has shown that incorporating TiO_2 nanoparticles into scaffolds can enhance the adhesion of osteoblast cells to

these structures [17]. This study investigated the use of a novel nanocomposite scaffold, PLA-g-P (HEM-co-NIPAAm)/TiO₂/HA nanocomposite, for repairing bone defects in rabbits (radius). This nanocomposite provides a smart, biomimetic scaffold with mechanical strength, thermoresponsive injectability, and osteoconductive mineral chemistry, making them highly effective for repairing critical-size bone defects. Based on the available literature, the fabrication and use of a PLA-g-P(HEM-co-NIPAAm)/TiO₂/HA nanocomposite for bone defect repair has not been previously investigated. This study therefore provides the first demonstration of its feasibility and regenerative potential.

2. Methods and materials

2.1. Study animals

This study was conducted on nine male New Zealand White rabbits weighing 1.8-2.5 kg. The animals used in this study were obtained from the Animal Breeding House of the Pasteur Institute of Iran (Karaj, Iran). The rabbits were housed individually in specialized cages under controlled environmental conditions at the Research School of Biological Sciences at Tabriz University. The animals were maintained in the animal house for one week for acclimation to new conditions before initiating the experiments. Throughout the experiment, the rabbits were maintained at a temperature of 22°C with a 12-hour light-dark cycle. They were fed a diet consisting of lettuce, alfalfa, cabbage, and bread twice a day (morning and evening) and had unrestricted access to water until the end of the study. The procedures were carried out based on the guidelines of the Ethics Committee of the International Association for the Study of Pain (Washington, DC, USA).

All experiments were approved by the Research Ethics Committee of University of Tabriz, Tabriz, Iran. All possible measures were taken to minimize animal suffering and distress throughout the experimental period.

2.2. Preparation of nanocomposites

2.2.1. Preparation of TiO₂/HA nanocomposite

Tetraisopropyl orthotitanate (TTIP) was used as the titanium source. By dissolving TTIP (5.0 mL) in ethanol (30 mL) and adding 0.75 mL chelator, glacial acetic acid, under magnetic stirring to prepare a Ti sol. Controlled hydrolysis was achieved by dropwise addition of a water/ethanol mixture at 5–10 °C; the resulting translucent solution was stirred for 1 h. Hydroxyapatite (HA) was synthesized separately by co-precipitation from Ca(NO₃)₂·4H₂O and P₂O₅. In the first step, P₂O₅ was slowly added to deionized water under ice-bath conditions to generate an H₃PO₄ solution. The H₃PO₄ solution was then added dropwise to the Ca(NO₃)₂ solution under vigorous stirring at pH ≈ 10 (maintained with NH₄OH) to obtain a Ca/P ratio of ~1.67. The Ti sol was then added slowly to the wet HA slurry and the mixture aged 12 h at room temperature, washed to neutral pH, dried at 80 °C overnight, and calcined in air at 500 °C for 2 h to yield the TiO₂/HA nanocomposite (named as TA). Phase composition was analyzed using powder X-ray diffraction (Cu K α radiation, 2 θ range of 20–70°), while surface morphology and elemental composition were investigated by scanning electron microscopy (SEM) equipped with energy-dispersive X-ray spectroscopy (EDX).

2.2.2. Hydrogel synthesis method

PLA-g-P(HEMA-co-NIPAAm) copolymer was prepared according to our previous study [18]. Briefly, the macromonomer was prepared by melting lactide at 130 °C under nitrogen, followed by addition of HEMA. Sn(Oct)₂ (0.05 wt%) was added as the catalyst, and the mixture was stirred at 130 °C for 5 h to complete the ring-opening polymerization. The resulting PLA-g-HEMA macromonomer was purified. For copolymerization, the macromonomer was dissolved with benzoyl peroxide and mixed with N-isopropyl acrylamide (NIPAAm). Free-radical polymerization was then carried out to obtain the PLA-g-P(HEMA-co-NIPAAm) copolymer. The as prepared copolymer was dissolved in phosphate-buffered saline (PBS) and named as hydrogel (H). The polymer component was confirmed by Fourier transform infrared (FT-IR) spectroscopy.

2.2.4. Preparation of cylindrical PLA-gP (HEM-co-NIPAAM)/TiO₂/Hydroxyapatite nanocomposite scaffold

Cylindrical nanocomposites were prepared using PLA-g-P(HEMA-co-NIPAAm)/HA and PLA-g-P(HEMA-co-NIPAAm)/TiO₂/HA. TiO₂/HA nanocomposite or HA (1.50 g) was dispersed in 5 mL by probe sonication (total 8 min, pulsed, ice bath). The copolymer (3.50 g) was dissolved in 10 mL phosphate-buffered saline and the nanoparticle suspension was added dropwise under vigorous stirring to prepare homogeneous suspensions and named as AH and TAH. The resulting homogeneous suspension was cast into silicone molds (8 mm Ø diameter × 10 mm height), thermally gelled at 37 °C for 15 min, and frozen at −80 °C for 4 h. The frozen constructs were lyophilized (primary drying −40 °C to −20 °C, secondary drying to 20 °C) to produce porous cylinders. Where indicated, methacrylate groups were photopolymerized in-mold (Irgacure 2959, 0.4 wt%; UV 365 nm, 3 min) prior to freezing to enhance mechanical

integrity. Before *in vivo* implantation, the scaffold was rehydrated, and sterilized by gamma irradiation (25 kGy). The cylindrical PLA-gP (HEM-co-NIPAAM)/Hydroxyapatite was named as CAH, and the cylindrical PLA-gP (HEM-co-NIPAAM)/TiO₂/Hydroxyapatite was named as CTAH (Fig 1A(s)).

2.3. Surgical procedure

2.3.1. Preoperative measures

Both hands of the rabbits underwent surgery to access the radius bone. The rabbits were randomly divided into three experimental groups, each consisting of three rabbits (6 radius). The first group, called the control group (Group 1), no implant, consisted of 6 radius samples. In the second group (Group 2), six radial defects were treated with the CAH nanocomposite. In the third group (Group 3), six radial defects were treated with the CTAH nanocomposite.

Before starting the surgery, all hair on the forearm area was completely shaved. Anesthesia was performed using a combination of xylazine (5 mg/kg), ketamine (40 mg/kg), and acepromazine (1 mg/kg) [19]. The surgical site was disinfected using a 7.5% povidone-iodine (Betadine®) solution following standard aseptic procedures. The preparation process for all rabbits was similar, and enrofloxacin (20 mg/kg/Sc) was used as a preventive antibiotic before surgery.

2.3.2. Surgical technique

The animals were anaesthetized by intramuscular injection of xylazine HCl (5 mg/kg, Alfasan, The Netherlands) and ketamine HCl (40 mg/kg, Alfasan, The Netherlands) and a 3-cm

longitudinal incision was made on the cranio-medial surface of the radius in both forelimbs. The diaphysis of the radius was exposed and a 1-cm fragment was removed to create a critical-sized defect. The pre-shaped nanocomposite scaffold was carefully fitted into the defect (Figure 1).

The muscles were closed using simple interrupted sutures, and the skin was closed with cross-mattress sutures. The surgical site was then dressed and bandaged according to standard procedures (Figure 3). After surgery, the rabbits were kept individually in the primary animal care center. For postoperative pain management, meloxicam was administered subcutaneously at a dose of 0.5 mg/kg twice daily for three days. A prophylactic antibiotic, enrofloxacin, was administered subcutaneously at a dose of 20 mg/kg once daily for five days.

Figure 1

2.4. Assessments Characterization

2.4.1. Characterization of Nanocomposites

The FT-IR spectrum recorded was using a Bruker Tensor 27 spectrometer. The samples were prepared as KBr pellets and the spectra were recorded in the range of 400–4000 cm^{-1} . The surface morphology of the TAH nanocomposite was examined by scanning electron microscope (Tescan MIRA3 FEGSEM and FEI Quanta 200).

2.4.2. In vitro cytotoxicity assay

The effect of nanocomposites (AH and TAH) on mesenchymal stem cell proliferation was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, a standard colorimetric method for determining cell viability. Human Dental Pulp Stem Cells (DPSCs) were obtained from Shahid Beheshti University Cell Bank and cultured in 75 mL culture flasks. For the assessment of vital cells at 1, 3, and 5 days, three 96-well plates were designated, all wells were seeded with 7,500 DPSCs, and the plates were incubated at 37 °C with 5% CO₂ and 50% humidity. The culture medium was refreshed every 3 days. Different concentrations of AH and TAH nanocomposites including 25, 50, 75 and 100 µg/mL were prepared and placed in the wells. After incubation, the supernatant was carefully discarded and the formazan crystals were dissolved using dimethyl sulfoxide (DMSO). The absorbance at 570 nm was measured using a microplate reader. Cell viability was expressed as a percentage of the control group.

2.4.3. Clinical assessment

During a five-week period after surgery, the rabbits underwent daily clinical assessments to monitor their general health status. Parameters including appetite, limb use, weight changes, and survival were systematically recorded. In addition, surgical sites were carefully evaluated and important informations were documented for any signs of swelling, inflammation, wound infection, or suture line opening.

2.4.4. Pathological evaluation

After 35 days following the surgery, animals were euthanized using sodium thiopental with the dosage of 50 mg/kg, IV, Sandoz, Austria BW with the intra venous injection, the radius bone was extracted and transferred to 10% neutral buffered formalin and reffered to the pathology laboratory. Different staining techniques were employed. Masson's trichrome staining was used to examine the formation and arrangement of collagen fibers. Hematoxylin-eosin staining was utilized to assess the extent and type of bone defect regeneration, as well as the bone formation process. Lastly, Alizarin red S staining was employed to determine the concentration of calcium deposits within the tissue. Using these staining methods and conducting a thorough histopathological examination, we gained valuable information about the bone's healing process, the organization of collagen fibers, the regeneration of bone defects, and the deposition of calcium. Microscopic slides were studied using a light microscope (A. Kruss-ML2100-Germany) and photomicrographs were taken from them Using a special camera (MD-130-China)

2.4.5. Radiological evaluation

Lateromedial and craniocaudal radiographs of the right and left fore limbs were taken using a digital radiology device (Mobile Drive AR30, SMAM, Muggio, Italy). The radiographs were taken on the day after the surgery and the 35th day after the surgery. The radiographs were reviewed to assess the degree of healing at the defect site using a grading system derived from the modified method of Lane and Sandhu [20], based on three healing signs: bone formation, union, and the degree of remodeling (Table 1).

Table 1

3. Results

3.1. physical properties of nanocomposite Drug evaluation results

3.1.1. FT-IR evaluation results

The FT-IR spectrum of the final hydrogel shows distinct peaks. For example, a peak at 1735 cm^{-1} was related to the stretching vibrations of carbonyl ester groups. A broad peak was located between 3310 cm^{-1} and 3434 cm^{-1} , indicating the presence of the N-H group, specifically associated with the NIPAAM polymer. Other notable peaks occur at 1646 cm^{-1} and 1383 cm^{-1} , corresponding to the vibrations of the amide and C-H groups, respectively [18].

3.1.2. FE-SEM/EDX evaluation results

FE-SEM image of TAH nanocomposite is shown in Fig.2. Hydroxyapatite and TiO_2 particles with semi-spherical shape are presented in the image. The agglomeration of the particles on the polymer are observed.

Figure 2

EDX of nanocomposite is presented in Table 2. The weight percentage of particles and elements is presented completely quantitatively, the weight percentage of C, O, P, and Ca indicates the presence of hydroxyapatite nanoparticles. In this table, the percentage of TiO_2 inside the hydroxyapatite nanocomposite is about 4.78%.

Table 2**3.1.3. MTT-assay evaluation results**

As can be seen in the diagram, the obtained hydrogel is completely safe and has no toxicity. In addition, due to the properties of the scaffold, the amount of cell growth on it is higher than in the control group, and with the increase in the concentration of the hydrogel, the amount of cell growth increases significantly, and this indicates that this hydrogel can be used as a scaffold for bone formation (Fig. 3).

Figure 3**3.1.4. Clinical evaluation results**

In the surviving animals, there was no swelling, redness, discoloration, or temperature changes, and no suture dehiscence was observed at the surgical site.

3.1.5. Pathological evaluation results**3.1.5.1. Hematoxylin-eosin staining**

Hematoxylin-eosin staining demonstrated a clear progression of bone healing across the experimental groups. In the control group, the repair process was in its early phase, as evidenced by abundant cartilage tissue, a high density of cells within lacunae, and poorly organized bone trabeculae. In CTAH nanocomposite group, Collagen fibers are more than the

other two groups. The number of fibrocyte and fibroblast cells decreased and the matrix was less than the other two groups. There are more collagen fiber bundles than both previous groups and their organization is better. The number of osteoblasts in the bone trabeculae has a moderate density, and the cartilage is less than the other two groups. Hyperemia was also present in the reparative tissue, while osteoblastic activity was observed at a moderate level. In the nanocomposite group without titanium dioxide, the healing process had advanced beyond that of the control group; however, progenitor cells remained relatively numerous, and trabecular organization was still incomplete. By contrast, in the nanocomposite group containing titanium dioxide, the bone trabeculae were arranged in a more regular pattern, the central region contained fewer progenitor cells, and the repaired tissue exhibited a more mature histological appearance, indicating a more advanced stage of bone regeneration (**Figure 4**).

Figure 4

3.1.5.2. Masson's Trichrome Staining

Masson's trichrome staining revealed distinct variations in collagenous matrix organization, remnant fibrocartilage, and the degree of osteogenesis across the experimental groups. In the control group, the reparative tissue was primarily fibrocartilaginous, with evidence of only nascent ossification. In the CAH group, bone trabeculae were more prominently developed compared to the control group. This group exhibited an increased density of collagen fibers with improved spatial orientation; however, a significant proportion of cartilage tissue persisted within the defect site. In contrast, the CTAH nanocomposite group showed a substantial

reduction in cartilaginous tissue, with the majority of the central defect area being replaced by mature bone. The trabecular architecture in this group was highly organized, indicating a more advanced stage of endochondral ossification and effective tissue remodeling (**Figure 5**).

Figure 5

3.1.5.3. Alizarin Red S Staining

Alizarin Red S staining revealed a progressive increase in mineral deposition and calcium accumulation within the reparative tissue across the studied groups. In the control group, calcium deposition was minimal, reflecting an early and immature stage of mineralization. The CAH nanocomposite group exhibited a higher degree of mineralized areas on the bone trabeculae compared to the control group, indicating enhanced osteoblastic activity and mineral recruitment. In contrast, the CTAH nanocomposite group demonstrated the most extensive and uniform calcium deposition. The relatively homogenous distribution of calcium across the trabecular structures in this group suggests a significantly higher degree of bone formation. According to the image, calcium has been deposited to some extent on the bone trabeculae. Osteoblast cells are abundant in the trabeculae mineralization and superior quality of the newly formed bone matrix compared to the other groups (**Figure 6**).

Figure 6

3.1.6. Results of radiological evaluation

Lateromedial radiographic views demonstrated the defect site more clearly than craniocaudal views. Of the six radii included in the control group, four cases survived until the end of the study. In these four cases, continuity of the radius at the defect site was maintained. Final-day radiographs of the remaining cases revealed an ulnar fracture at the level of the radial defect in two cases. This fracture resulted in approximately 90° cranial rotation of both the radius and ulna at the defect site, leading to malunion (Fig. 7). In the remaining case, cranial displacement of the distal radius disrupted bone continuity on both sides of the defect; however, bone formation at the defect site was still observed (Fig. 7).

Figure 7

In CAH nanocomposite group (group 2), of the 6 cases included in the group, 4 cases survived until the end of the study. In them, the continuity of the radius at the defect site was maintained. Radiographs of the last day showed cranial displacement of the distal radius and disruption of bone continuity in 3 cases, and maintenance of bone continuity in one case. However, bone formation at the defect site was considerable in two cases, and callus formation on the ulna was observed in all 4 cases, which may be due to its irritation during surgery (Fig. 8).

Figure 8

In CTAH nanocomposite group, out of 6 cases, 4 cases survived until the end of the study. In the survived cases, the continuity of the radius bone at the defect site was maintained.

Radiographs of the last day of the survived 4 cases showed cranial displacement of the distal radius in 1 case. Ossification at the margin of the distal part of the radius and increased opacity of the defect site due to ossification was observed in all four cases (Fig. 9).

Figure 9

It was found that 50% of the cases had fractures in the ulna. Out of 12 cases that reached the end of the study, 6 cases experienced displacement of the distal of the radius, which resulted in a loss of continuity of the radius bone at the defect site. A comparison of ossification score at the defect site in cases which had the highest percentage of bone continuity maintenance showed that ossification in group 3 (CTAH) was higher than in group 2 (CAH) and group 1 (control).

Complete union and reconstruction were not observed in any of the cases. Ossification at the margin of the distal bone defect of the radius was mainly observed bone production. Also, in all radiographs of the last day, degrees of ossification were observed on the ulna bone at the site of the radius defect.

Considering the results of the histopathological and radiological evaluation, it can be inferred that the ossification of the hydroxyapatite + titanium dioxide nanoparticles group is higher than the hydroxyapatite and control groups, and the amount of cartilage formation has decreased. Also, according to the evidence in the samples stained with Alizarin red S, the amount of calcium deposition in the hydroxyapatite + titanium dioxide nanoparticles group is higher than in the hydroxyapatite and control groups. Furthermore, according to the evidence in the

samples stained with Masson's trichrome, the amount of collagen fiber formation and their organization in the hydroxyapatite + titanium dioxide nanoparticles group is higher than the hydroxyapatite and control groups. The nanocomposite used in the recent study can be a suitable option for bone defects. However, further studies are needed for clinical use.

4. Discussion

Combined FT-IR, SEM, and EDX results show that TAH scaffold has suitable chemical properties, crystal structure, porous morphology, and elemental composition. The combined use of TiO₂ and hydroxyapatite in a biocompatible hydrogel (PLA-g-P(HEMA-co-NIPAAm)) enhances the mechanical and biological properties of the scaffold. This makes it a promising option for bone tissue engineering and regenerative medicine applications.

The MTT assay results demonstrate that the AH and TAH nanocomposites exhibit excellent cytocompatibility, with no evidence of cytotoxic effect toward the tested cells, making it suitable for biomedical uses. Notably, a significant increase in cell growth on the hydrogel scaffold compared to the control group, indicating that the nanocomposites not only support cell survival but also promote cellular metabolic activity [18, 21]. These include their porous structure, ability to hold water, and features that mimic natural extracellular matrix. These qualities help nutrients move easily, remove waste, and improve cell interactions with the materials. The increase in cell growth with higher nanocomposites content suggest that more hydrogel provide better support and more sites for cells to attach and multiply. Overall, these results indicate that these nanocomposites could be effective scaffolds for bone grafting and regeneration.

In recent years, there has been a significant amount of research focused on finding alternatives to use autologous grafts for bone grafting. Autologous grafts are associated with various issues such as pain, high cost, delayed organ use, and the risk of complications like infection, bleeding, and even death [22]. One important method that has been explored is the use of scaffolds made from biocompatible materials to repair damaged tissues, including fractures and bone defects. These scaffolds create an ideal environment for cell growth and the transfer of biomolecules, essentially acting as artificial tissues to repair damaged areas in the body [23, 24]. However, it is crucial to prevent bacterial and fungal contamination in the scaffold growth environment to ensure successful tissue repair. Contamination can occur through the invasion of harmful agents from the outside environment or the presence of bacteria growth compounds in the scaffold itself, such as starch or gelatin. Any contamination during scaffold construction or accidents and open wounds can fail when it comes to tissue transplantation [25, 26]. Therefore, it is very important to develop strategies that prevent contamination and ensure successful grafting and tissue repair. In some samples of this study, the fractured parts of the radius were moved and the bone alignment was not maintained until the end of the study. Additionally, moving and transporting animals for radiological imaging may have caused movement of the broken parts of the radius. This study utilized a PLA-gP (HEM-co-NIPAAM)/TiO₂/Hydroxyapatite nanocomposite (as a physical scaffold for healing bone defects in rabbit (radius) for the first time. Out of 18 rabbits in the study groups, 9 rabbits (50%) experienced a fracture in the ulna, which resulted in a misalignment of the radius bone at the defect site. Moreover, creating a gap in both hands' radius, which doubles the pressure on the lower ulna, could be a cause of its fracture. In some cases, bone alignment was not maintained throughout the study period. In the models where there were bone defects in the radius, the ulna bone acts as a support for the radius and there is no need for external stabilization such as

casting [27]. As rabbit's Haversian system is similar to humans, it is a good experimental model for conducting studies to investigate the fracture healing process [28]. However, in the present study, in several samples, displacement of the fractured pieces of the radius occurred and the alignment of the bone was not maintained until the end of the study. It is likely that in 1 cm long defects in the radius if external fixation is used, the alignment of the bone will be better maintained. Evaluation of the qualitative histopathological results showed that the formation of bone lamellae and calcium deposition in TAH nanocomposite group increased more than both the control and AH groups. Also, in terms of organization and orientation of collagen fibers, TAH nanocomposite group performed better than both the control and AH groups, and the lowest amount of cartilage formation belongs to the TAH nanocomposite group, which indicates that better bone formation has taken place. In a study by Oryan et al., histopathological examinations and radiographic images revealed that the gap in the empty and normal saline group was filled with fibrous tissue. This is why when a bone defect is filled with fibrous tissue, it no longer allows bone formation by filling with fibrous tissue. For this reason, in the Trancron group, less fibrous tissue is formed due to the properties of Trancron, so the rate of bone formation is higher in the Trancron group [29]. In the present study, the amount of cartilage and fibrous tissue formation at the defect site in the nanocomposite group along with titanium dioxide performed better than both the control and non-main treatment groups. Meimandi Parizi et al. [30] stated in their research that hydroxyapatite plays an effective role in accelerating bone repair. In addition, it helps the natural and physiological construction of the treated site, but its effect on internal bone growth is still unknown. Also, in this study, it is reported that a significant formation of new bone in the hydroxyapatite group compared to the control group. Other reasons for the rapid effectiveness of hydroxyapatite include its angiogenic properties, bone formation guidance, and the mechanism of creating a strong and

rigid mechanical structure in the fracture area and rapid fracture repair. It should be noted that if the size of the bone fracture site is large, hydroxyapatite has no effect on filling the defect and prevents the defect site from being filled by forming fibrous or fibrinous-cartilaginous tissue. New bone formation was observed at the fracture edges in direct contact with hydroxyapatite, indicating the osteogenic properties of hydroxyapatite and its ability to guide bone regeneration at the defect interface. However, the formation of new bone at the edges is not sufficient to fill the gap and connect the two sides of the fracture together [30]. In another study, Sahmani et al. synthesized hydroxyapatite/titanium dioxide nanocomposite scaffolds with different percentages of titanium dioxide. The aim of fabricating scaffolds with different percentages of titanium dioxide was to accurately simulate the properties of bone in terms of microstructure, morphology, and mechanical properties. The results indicated that the fabricated three-dimensional nanocomposite scaffolds have a highly interconnected porous structure. Also, this sample has a minimum pore size (70-130 nanometers) and porosity percentage (87%). Swelling experiments showed that increasing the percentage of titanium dioxide in the structure of three-dimensional nanocomposites leads to less swelling behavior during the test period. The sample containing the maximum percentage of titanium dioxide showed suitable mechanical properties compared to trabecular bone tissue. The MTT assay on L929 cells indicated that scaffolds with different percentages of titanium dioxide are non-toxic and have suitable biocompatibility. As a result, the newly introduced scaffolds are a suitable candidate for use in bone tissue engineering [31]. Javanmardi et al. inferred from their findings that titanium dioxide nanocomposite, due to the production and proliferation of fibroblasts, stimulation of angiogenesis, and creation of granulation tissue in the early days of healing. Furthermore, the nanocomposite accelerated the repair process by increasing wound contraction and reinforcing the injured area through improved organization and alignment of

granulation tissue and collagen fibers. In addition, through the antimicrobial effect of titanium dioxide nanocomposite, it can prevent tissue infection [32]. As a result, the present study indicates that TAH scaffold is a suitable scaffold for healing and bone growth around the implant. In the study by Rahimzadeh et al., on 18 adult male New Zealand rabbits, a 1 cm piece of the radius was removed, and the defect was fixed with a small metal plate with 4 screws. Two weeks after surgery, the limb was cast. According to the results of this study, the transplantation of cancellous bone scaffold coated with nanohydroxyapatite has a positive effect on the complete and qualitative formation of callus at the defect site [33]. The total score of bone proliferation at the defect site was higher in group 2 than in groups 1 and 3. Radiological images showed that this was due to bone formation from the ulnar cortex at the defect site. It seems that the destruction of the ulna during surgery has led to bone formation in this part of the bone. Also, the highest number of surviving animals was in this group, which affected the higher score of bone proliferation. The recent study had some limitations. However, the turning point of the recent study was radiological evidence indicating that in cases of maintaining bone continuity and nanocomposite at the defect site, bone formation in group 3 (hydroxyapatite + titanium dioxide nanoparticles) was higher than in group 2 (hydroxyapatite) and group 1 (control), which is consistent with the findings of similar studies in this field. This seems to be due to the positive effects of the nanocomposite placed at the defect site. Supporting this idea, a study conducted by Killin in 2008 also confirmed the significant role of hydroxyapatite in accelerating the process of bone healing. The use of hydroxyapatite was found to promote the formation of new blood vessels at the defect site, leading to the development of thicker pseudo-sinusoidal areas, up to four times thicker compared to the empty control group [34]. Radiographic examination in a study by Ronagh et al. on the radiographic evaluation of the healing process of mandibular bone defects with a

combination of platelet-rich gel (PRP gel) and hydroxyapatite nanoparticles (NanoHA) in dogs showed that although there was no significant difference between the NanoHA+PRP gel group and the control group, the onset and continuation of the healing process in the NanoHA+PRP gel group was faster and greater than the control group. Therefore, the combination of nanohydroxyapatite can probably be a suitable substitute for filling bone defects [35]. In a study by Chen, a rabbit model was used to evaluate the osteogenesis of 3D composite scaffolds of polylactic acid/nanohydroxyapatite (PLA/nHA). All these results indicate that 3D-printed PLA/nHA scaffolds have great potential for repairing and reconstructing large bone defects [36]. Furthermore, in the study by Velioglu and colleagues, poly-lactic acid scaffolds were fabricated using 3D printing technology and were tested for their biomechanical properties and porosity. The biomechanical properties were compared with trabecular bone samples taken from cattle, and the results showed that, due to the similarity between PLA scaffolds and trabecular bone in terms of pore size, porosity, and mechanical properties, the 3D-printed PLA scaffolds in this study can be considered as replacement options for bone repair and reconstruction [37].

Conclusion and Future Perspective

The nanocomposite used in this study, particularly the combination with titanium dioxide nanoparticles, showed promising results for the repair of bone defects. The positive effects of this nanocomposite combination on bone regeneration were evidenced by the histopathological and radiological evaluations. However, further studies are needed to fully establish the clinical potential of this scaffold for bone tissue engineering applications.

CRedit authorship contribution statement

D. A. and E. J. S. Performed the animal experiments and data collection. H. M. S. and D. H. M. critically revised the manuscript for important intellectual content. S. J. designed and performed the animal experiments, B. D. conceived and designed the study. A. A. S. performed the histopathological examinations and histomorphometric analyses, S. M. H. performed the radiographic/X-ray examinations, micro-CT imaging and quantitative analyses. All authors contributed in writing and reviewing the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data supporting this article have been included as part of the main manuscript.

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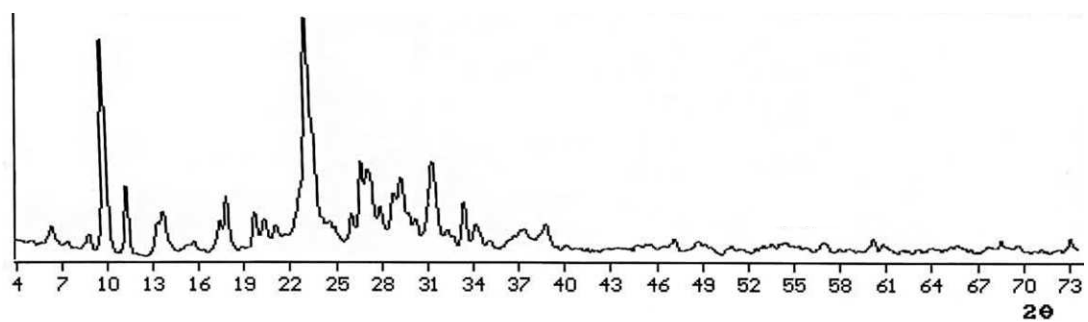


Figure 1: XRD pattern of TiO₂/NZ/Chit nanocomposite

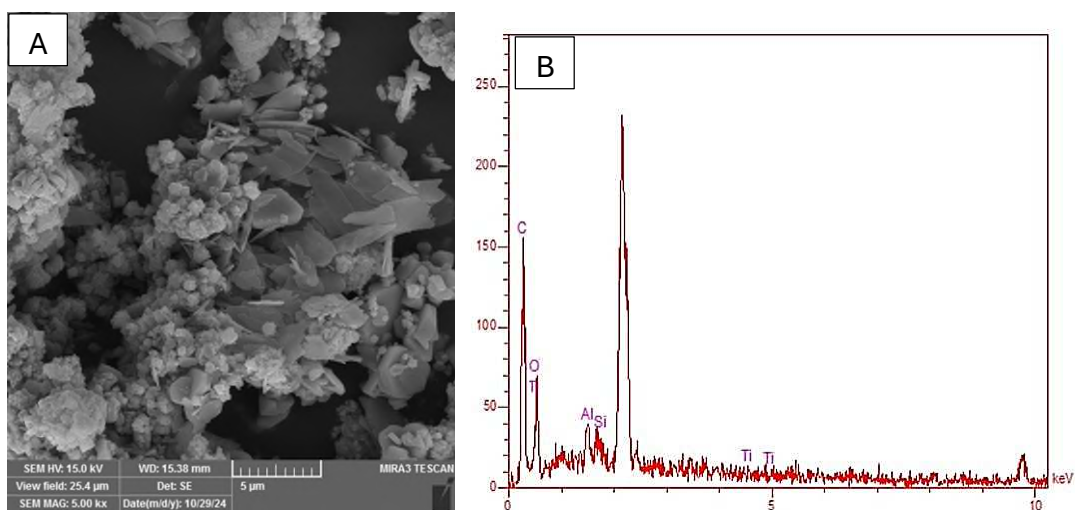


Figure 2: A) Scanning Electron Microscopy Image, B) EDX spectrum of TiO₂/NZ/Chit Nanocomposite

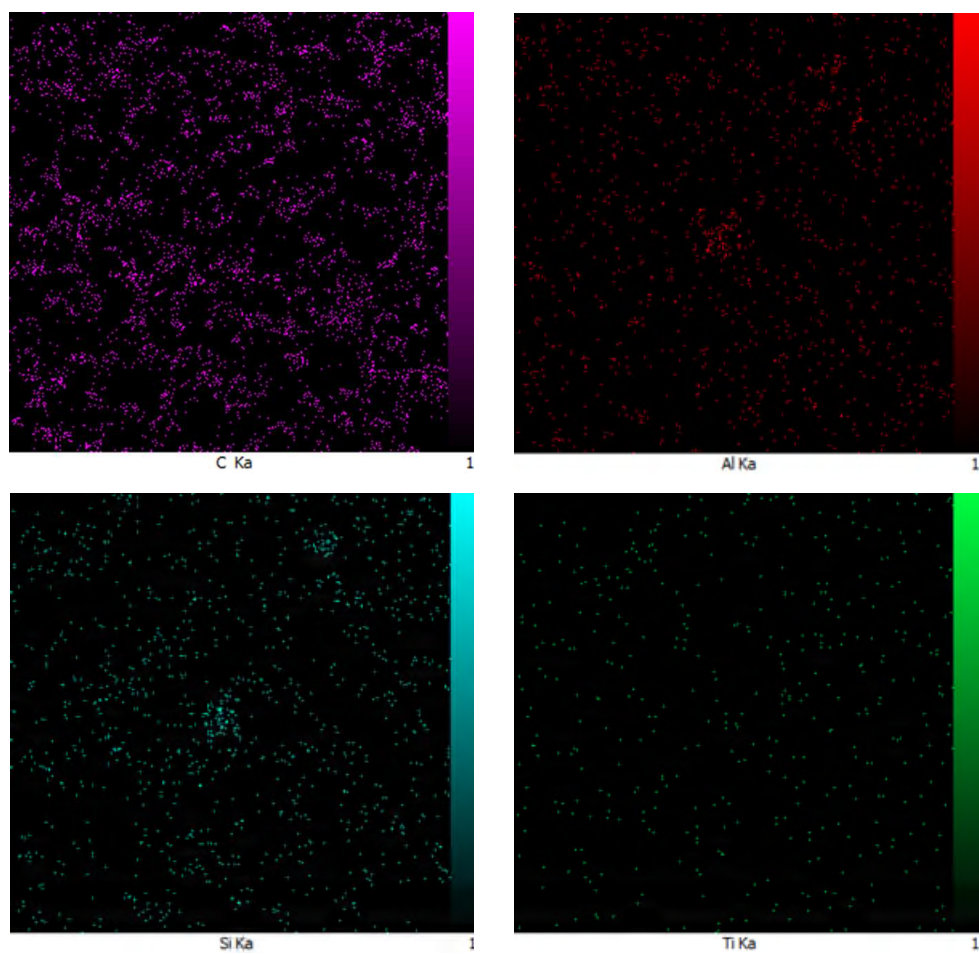
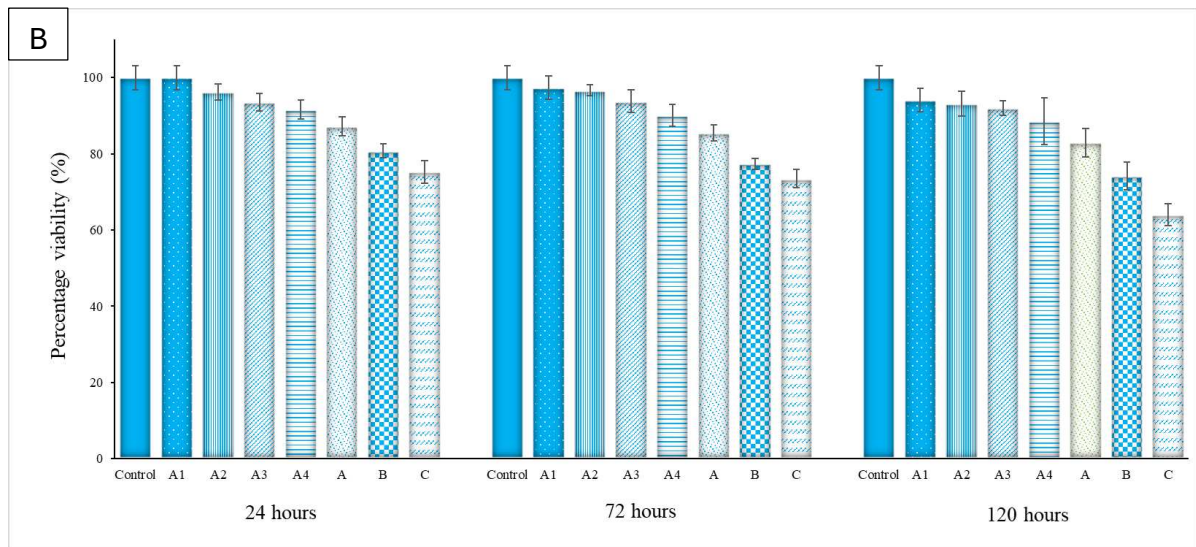
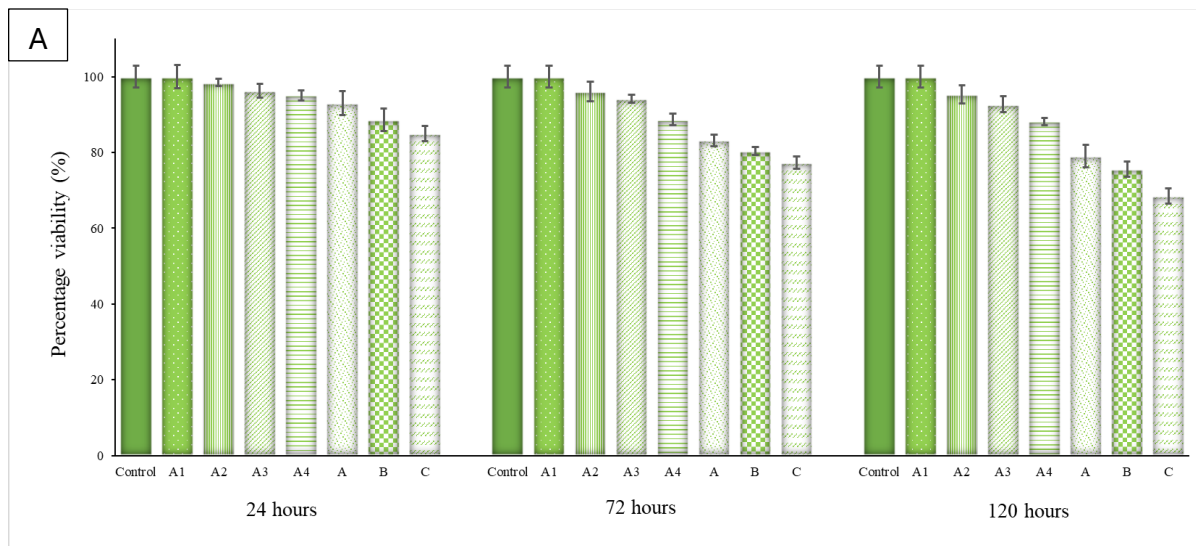


Figure 3: MAP analysis related to the acrylic resin containing $\text{TiO}_2/\text{NZ}/\text{Chit}$ nanocomposite



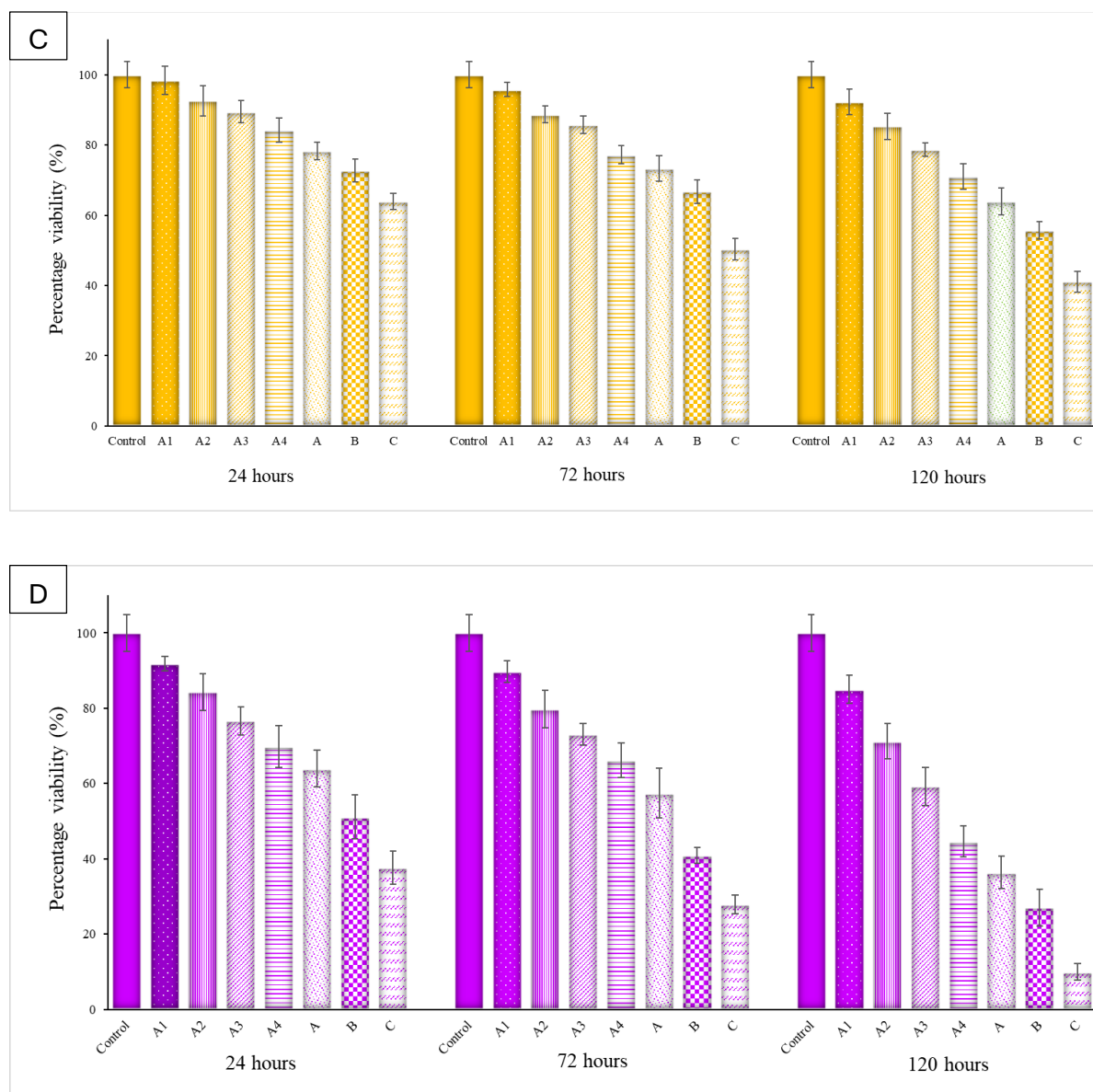


Figure 4: Cytotoxicity Assessment via MTT Assay; a) 50, b)100, c)150, and d)200 µg/mL

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