Pulp regeneration using a peptide nanofiber artificial scaffold on animal models: A preliminary study

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Abstract

Background. In regenerative endodontic procedures (REPs), it is crucial to find effective materials. This study introduces glycosaminoglycan (GAG) mimetic peptide amphiphile (PA, GAG-PA) and K-PA nanofibers, synthesized to emulate sulfated GAGs, aiming to enhance tissue repair within damaged pulp – an area where standardized protocols are currently lacking.

Objectives. The objective of this study was to investigate the regenerative potential of GAG-PA nanofibers in REP.

Materials and methods. Heparan sulfate mimicking PAs was designed to develop a bioactive nanofibrous supramolecular system. The cavities on the mesial surfaces of the first upper molars of 8 rats (4 rats in the study group and 4 in the control group) were prepared, and the pulps were perforated. Then, the material was applied onto the dental pulp, and the cavities were closed with a self-curing glass ionomer cement filling material. Physiological saline was used in the control group. Thirty days after application, the teeth were extracted, and the formation of regenerative tissue sections in the pulp was evaluated using hematoxylin and eosin (H&E) staining and Masson's trichrome staining.

Results. After 30 days, H&E staining demonstrated robust tissue regeneration in the implanted region, with minimal neutrophil infiltration. Masson's trichrome staining confirmed reparative dentin formation. Quantitative analysis revealed a regeneration percentage of 85% in the study group, compared to 80% in the control group. Statistical analysis showed no significant difference in regeneration between the groups ($p > 0.05$).

Conclusions. Our comprehensive study, utilizing GAG-PA and K-PA nanofibers, demonstrated successful synthesis, characterization and formation of nanofiber networks. The in vivo experiment with rats exhibited substantial tissue regeneration with quantifiable results supporting the efficacy of the nanofiber approach. Statistical analysis confirmed the consistency between the study and control groups, emphasizing the potential of these nanofibers in endodontic tissue regeneration applications.

Key words: regenerative endodontics, dental pulp, tissue scaffolds

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Background

The dental pulp is a vital soft connective tissue that has the remarkable ability to generate dentin in response to external stimuli. Furthermore, it plays a crucial role in maintaining the biological and physiological vitality of dentin, thus contributing to dental homeostasis. Dental pulp inflammation, referred to as pulpitis, is an inflammatory dental condition characterized by the degradation of dental pulp, leading to the eventual loss of its functionality. The entry of bacteria and their harmful components into the pulp triggers an inflammatory response within various host cells, such as dental pulp cells, macrophages and other immune cells.[1] Nevertheless, these actions impede the inherent self-repair mechanism of the dental pulp, resulting in prolonged inflammation that is immensely destructive and can potentially lead to tissue necrosis.[2] In the majority of cases, complete removal of the pulp tissue is performed during root canal treatment, even if a significant portion of the pulp remains healthy.[3] Unfortunately, this procedure leads to the loss of dentin, escalating the risk of tooth fractures and ultimately culminating in the extraction of the tooth. However, the possibility of extraction can be averted if the damaged pulp tissue can be regenerated.

Regenerative endodontic procedures (REP) have emerged as a viable option for addressing immature necrotic teeth with apical periodontitis, aiming to rejuvenate the necrotic pulp and facilitate root development. The absence of a standardized protocol for this treatment method has led the American Association of Endodontists (AAE) and the European Society of Endodontology (ESE) to advocate for a shared clinical guideline.[4–6] Key distinctions in these procedures typically revolve around the application of intracanal media, the method of inducing bleeding, the concentration of sodium hypochlorite, and the use of biological matrices.[6] This collaborative guideline serves as a foundation, allowing flexibility in clinical approaches while maintaining a common framework for REP.

The utilization of stem cells and/or biomaterials for dental pulp regeneration is regarded as a crucial approach to preserving tooth health. Before clinical implementation, it is essential to conduct animal studies to validate the bioavailability, safety and effectiveness of novel treatment modalities involving stem cells or biomaterials in the context of dental pulp regeneration.[7]

Synthetic biomaterials that can mimic extracellular structures have proven to be invaluable for tissue engineering and regenerative medicine applications.[8,9] Extracellular matrix (ECM) mimetic materials can recognize and control cell movements and behavior, and 3-dimensional (3D) microenvironments created using such biomaterials enable stem cells to multiply and differentiate through tailored biological signs.[10]

The biomechanical properties of synthetic polymers are critical considerations in various biomedical applications, particularly in the field of tissue engineering. Engineered to mimic the structural characteristics of natural tissues, synthetic polymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers like poly(lactic-co-glycolic acid) (PLGA) offer versatility in tailoring mechanical strength.[11] Factors such as molecular weight, crystallinity and processing methods influence the biomechanical behavior of these polymers. The mechanical strength of synthetic polymers is essential for providing structural support during tissue regeneration.[12] Additionally, the biodegradability of these polymers, where controlled degradation over time matches tissue regeneration rates, is a crucial feature. The ability to fine-tune biomechanical properties makes synthetic polymers valuable in creating scaffolds and implants that can integrate seamlessly with the biological systems they aim to support and regenerate.

In nature, proteins in the intercellular structure, known as collagen, are used by cells for mechanical support and attachment.[13] The length of a collagen fibril, which consists of aggregated polypeptides, is approx. 300 nm, and the diameter is approx. 1.5 nm. Cells can interact with collagen through integrin proteins, such as fibronectin and laminin, and move within the intercellular structure. Previous studies have shown that Arg-Gly-Asp-Ser (RGDS) on fibronectin and Ile-Lys-Val-Ala-Val peptide sequences on laminin form vital binding sites for cells to use these proteins.[14–16] These short peptide sequences have thus been placed on many synthetic polymers, allowing cells to move in environments created by these polymers. However, in general, these polymers cannot naturally degrade and do not accurately portray biological signals.

Scaffolds, 3D frameworks designed to mimic the ECM, must possess specific biomechanical characteristics for successful integration with host tissues.[17] Mechanical support is a key consideration, with properties such as compressive strength, tensile strength and modulus of elasticity crucial in maintaining structural integrity. Porosity and pore size are also vital factors, influencing nutrient and oxygen diffusion, as well as facilitating cell ingrowth.[18] Biocompatibility is essential to promote cell attachment, proliferation and differentiation, while the dynamic balance of degradation and remodeling ensures that the scaffold degrades over time, synchronizing with the natural regeneration of the tissue. The ability to modulate these biomechanical properties makes scaffolds versatile tools in regenerative medicine, providing a customizable platform for tissue repair and replacement.[19]

Ongoing research focuses on the development of synthetic or natural scaffolds.[20] Various alternative scaffolds employed for pulp regeneration, such as soluble collagen, absorbable gelatin sponge and platelet-rich plasma, have undergone examination in animal models.[21–23] However, the histological findings from these studies consistently indicate unsuccessful healing. Furthermore, a study assessing the regenerative potential of 2 lyophilized chitosan-based scaffolds (hyaluronic acid : chitosan and pectin : chitosan) reported unfavorable histological results.[24] Up to now,

cross-linked collagen sponge scaffolds have been the sole scaffolds utilized, yielding more favorable outcomes.[25]

Composed of a network of blood vessels, nerves and fibrous tissues, dental pulp contributes to the mechanical resilience of the tooth. Its ability to resist compressive forces is essential for protecting the underlying pulp tissue from external impacts and occlusal forces during biting and chewing.[26] The elasticity of dental pulp allows it to absorb and distribute mechanical stresses, acting as a shock absorber and minimizing the risk of damage to the tooth. Additionally, the pulp's regeneration plays a role in maintaining tissue health and facilitating reparative processes. Understanding the biomechanical properties of dental pulp is essential for designing dental materials and procedures that preserve pulp vitality while ensuring the overall mechanical stability of the tooth structure.[27]

The use of animal models in research on REP is necessary for several reasons. These models provide valuable insights into the efficacy, safety and mechanisms of regenerative approaches for pulp treatment. Animal models allow researchers to evaluate the potential of REP to promote pulp regeneration and repair. For example, studies in rats or dogs can assess the effectiveness of different scaffolds, growth factors or stem cell-based therapies in stimulating pulp regeneration.[28] Animal models help researchers investigate the long-term outcomes of regenerative pulp therapies. By examining histological, radiographic and functional parameters, these models provide valuable data on the success of the treatment and the quality of the regenerated pulp tissue.[29] Animal models enable researchers to evaluate the safety and biocompatibility of regenerative materials and procedures used in pulp therapy. These models help identify potential adverse effects, such as inflammation, immunological reactions or neoplastic transformations, which are critical before translating therapies to human clinical trials.[30] Animal models provide a platform to study the underlying mechanisms involved in pulp regeneration. By analyzing the cellular and molecular events occurring during the regenerative process, researchers can gain a deeper understanding of the biological pathways and factors influencing pulp tissue repair.[31] In summary, animal models are essential for evaluating the potential of REP, assessing treatment outcomes, ensuring safety and biocompatibility, and understanding the underlying mechanisms of pulp regeneration. These models play a crucial role in advancing REP and ultimately improving clinical treatments for dental pulp-related conditions.

In this study, an attempt was made to overcome some of the limitations associated with traditional scaffolds. Peptide nanofiber artificial scaffolds were introduced and evaluated as an alternative in an in vivo animal study. The purpose was to explore the potential advantages of peptide nanofiber scaffolds as an innovative approach for pulp regeneration, acknowledging the ongoing need for advancements in this field.

The hypothesis of this study posits that the application of glycosaminoglycan (GAG) mimetic peptide amphiphile (PA, GAG-PA) nanofibers as a bioactive nanofibrous supramolecular system can promote pulp regeneration in rat molars with perforated pulps. Specifically, we hypothesize that the use of heparan sulfate mimicking PAs in the treatment of pulpal injuries, when compared to a control group treated with physiological saline, will result in a statistically significant increase in the percentage of regeneration within the damaged pulp area. The evaluation of regenerative tissue sections through hematoxylin and eosin (H&E) staining and Masson's trichrome staining is expected to reveal a measurable enhancement in reparative dentin formation in the study group. However, to establish statistical significance of these observed differences, it is further hypothesized that an increase in the number of animals in future studies will be necessary.

Objectives

The present study aimed to evaluate the effect of bioactive peptide nanofibrous hydrogels, which can create an artificial 3D environment, on the differentiation of dental pulp stem cells into odontoblasts and vascular nerve cells for pulp regeneration in animal models.

Materials and methods

Materials

The acbr (Karlsruhe, Germany) or Nova-Biochem (London, UK) supplied all the amino acids, such as lauric acid, 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin (Rink amide MBHA resin), diisopropylethylamine (DIEA), and 2-(1H-benzotriazol-1-yl)-1,1,3,3 tetramethyluroniumhexafluorophosphate (HBTU). Thermo Fisher Scientific (Waltham, USA) and Sigma-Aldrich (Darmstadt, Germany) provided the remaining chemicals used in this study.

Synthesis, characterization and purification of peptide amphiphile molecules

The Lauryl-Val-Val-Ala-Gly-Lys-Am (K-PA) and Lauryl-Val-Val-Ala-Gly-Glu-Gly-Asp (Lys-p-sulfobenzoate)-Ser-Am (GAG-PA) peptides were synthesized using a previously published procedure.[32] The Fmoc solid-phase peptide synthesis method was employed for their synthesis.

The synthesized peptide amphiphiles were subjected to liquid chromatography–mass spectrometry (LC–MS) analysis to determine their identity and purity. For LC–MS analysis, an Agilent 6530 Q-TOF instrument with an electrospray ionization (ESI) source and either a Zorbax Extend-C18 2.1×50 mm column (Agilent, Santa Clara, USA) (for basic conditions) or a Zorbax SB-C8 4.6×100 mm column (for acidic conditions) was employed to obtain

Table 1. The statistical values related to the regenerated area and the mean color intensity

Statistics	Regenerated area		Mean color intensity	
	serum	gel	serum	gel
median	85.86	92.1	45.06	68.04
SD	NaN	12.05	NaN	13.23
Minimum	85.86	71.43	45.06	54.79
Maximum	85.86	92.48	45.06	81.26
U	1		0	
z	–0.45		–1.34	
asymptotic p	0.655		0.18	
exact p	1		0.5	
r	0.22		0.67	

SD – standard deviation; NaN – not a number.

mass spectra. The mobile phase consisted of a gradient of water (with 0.1% formic acid or 0.1% ammonium hydroxide (NH_4OH) and acetonitrile (with 0.1% formic acid or 0.1% NH_4OH).

To purify the peptides, an Agilent preparative reverse-phase HPLC system with either a Zorbax Extend-C18 21.2×150 mm column (for basic conditions) or a Zorbax SB-C8 21.2×150 mm column (for acidic conditions) was utilized. The mobile phase employed a gradient of water (with 0.1% trifluoroacetic acid (TFA) or 0.1% NH_4OH) and acetonitrile (with 0.1% TFA or 0.1% NH_4OH). For the K-PA peptide, a 0.1 M hydrochloric acid (HCl) solution was used to remove residual TFA, followed by lyophilization. The details can be found in Table 1.

Visualization of peptide amphiphile nanofiber network using scanning electron microscopy

The morphology of nanofiber networks formed by peptide amphiphile molecules was examined using scanning electron microscopy (SEM) (FEI Quanta 200 FEG SEM; FEI Company, Hillsboro, USA). To prepare the samples, GAG-PA and K-PA solutions (10 mM) were mixed at a 1:3 ratio in a final volume of 60 µL on a silicon wafer, allowing for the stabilization of all net charges. After 15 min of gelation, the samples underwent sequential dehydration using ethanol concentrations of 20%, 40%, 60%, 80%, and 100% v/v. Subsequently, an Autosamdri-815B critical point dryer (Tousimis, Rockville, USA) was used for drying the samples. A thin coating of 4-nm gold-palladium (Au-Pd) was applied to the dried samples, and imaging was conducted using an FEI Quanta 200 FEG SEM (FEI Company) operating in high vacuum mode with a 5 keV beam energy.

Characterization of nanofiber secondary structures through circular dichroism analysis

To conduct circular dichroism (CD) measurements, samples were prepared by mixing 3×10^{-2} mM GAG-PA and 9×10^{-2} mM K-PA. The CD measurements were carried out using a JASCO J815 CD spectrometer (JASCO, Tokyo, Japan) at room temperature (21–22°C), covering a wavelength range of 300–190 nm. The data interval and data pitch were set at 0.1 nm with a scanning speed of 100 nm/min. Each measurement was performed in triplicate. The digital integration time (DIT) for CD measurements was set to 1 s, the bandwidth was set at 1 nm, and the sensitivity was set to the standard value.

Preparation of scaffolds

A bioactive nanofibrous supramolecular system was developed using peptide amphiphiles that mimic heparan sulfate (GAG-PAs) (Fig. 1,2). The GAG-PA and K-PA molecules were dissolved in double-distilled water (ddH_2O) at a concentration of 10 mM and subjected to sterilization under UV light for 1 h. To stabilize all net charges, the samples were prepared by mixing GAG-PA and K-PA solutions at a volume ratio of 1:3.

Ethics statement

The Animal Research Ethics Committee of Gülhane Military Medical Academy (GMMA; Ankara, Turkey) approved the clinical protocol on March 6, 2012, with reference No. 187. The experimental animals were provided by the GMMA within the project number AR-2012/55.

Animal model

Animal pilot studies related to REP are generally conducted on small animal models, and rats are commonly used. In this context, 8 female Sprague Dawley rats, weighing approx. 200 g each, were employed for the study when they reached 6 weeks of age. Our study was planned with 8 rats as it is a preliminary investigation. These rats were housed under standard conditions, with a temperature of 23°C and a regular light/dark schedule, and provided with unrestricted access to food and water. The cages were equipped with appropriate bedding material for comfort, and regular cleaning was performed to maintain hygienic conditions. All necessary measures were taken to ensure the wellbeing and ethical treatment of the rats throughout the experimental period. The overall health status of the rats used in the study was assessed through regular monitoring and evaluation, following established protocols used in similar studies. The rats were observed daily for any signs of distress, abnormal behavior or physical abnormalities. Body weight measurements were recorded periodically to monitor their growth and overall condition. Additionally, clinical parameters such as coat appearance, activity level and food and water intake were assessed to ensure their wellbeing. Any rats showing signs of illness or discomfort were promptly evaluated by a veterinarian, and appropriate measures were taken to address their

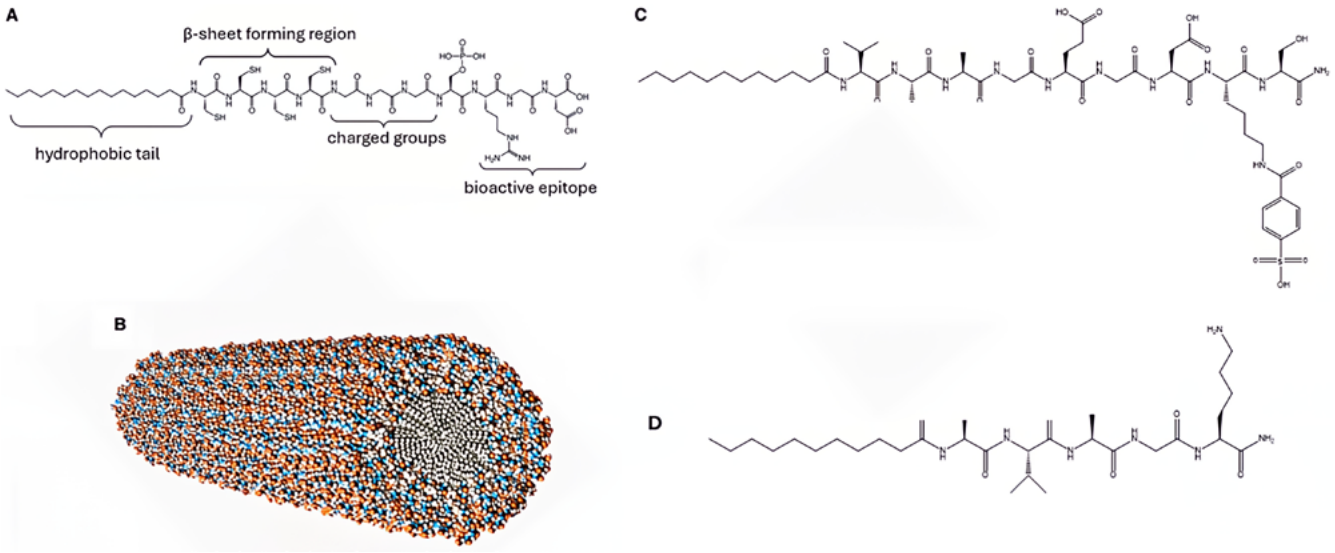

Fig. 1. A. Chemical composition of PA; B. Illustration depicting the self-assembly of PA molecules forming a cylindrical micelle. Chemical structures of (C) negatively charged GAG-PA and (D) K-PA

PA – peptide amphiphile; GAG-PA – glycosaminoglycan mimetic peptide amphiphile; K-PA – positively charged peptide amphiphile.

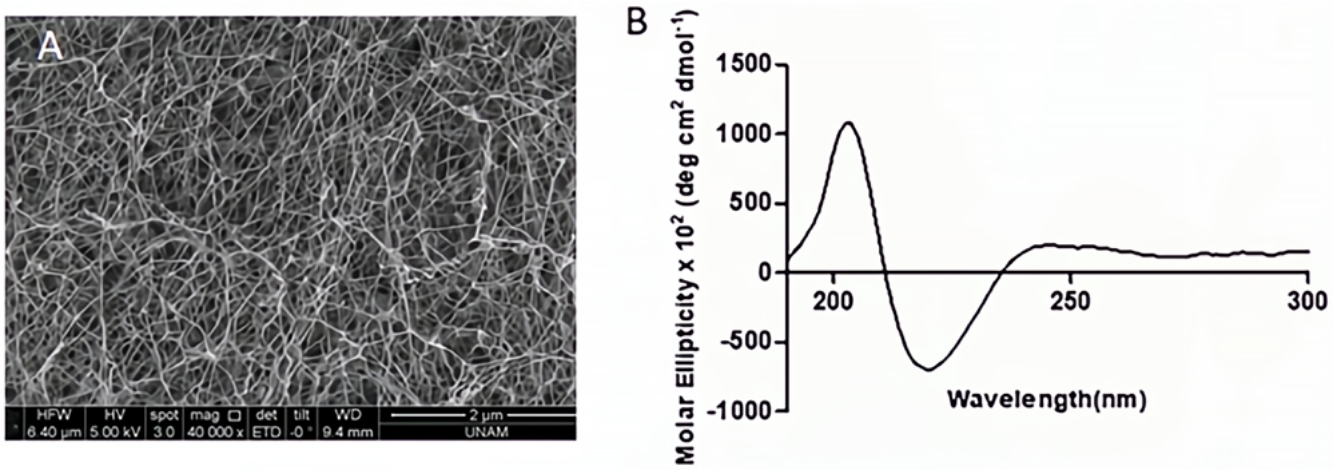

Fig. 2. A. SEM image of nanofibrous networks formed by GAG-PA/K-PA; B. CD spectra of GAG-PA

SEM – scanning electron microscope; GAG-PA – glycosaminoglycan mimetic peptide amphiphile; K-PA – positively charged peptide amphiphile; CD – circular dichroism.

health needs. An animal care technician provided daily care to the animals, maintained their living environment, and monitored their health and wellbeing.

Application procedure

To induce a pulp defect, the rats were administered intraperitoneal (ip.) injections of 2% xylazine and 10% ketamine for anesthesia. A total of 8 rats were included in the experiment and divided equally into 2 groups: a study group and a control group, with 4 rats assigned to each group. Gingival tissue and maxillary molars were disinfected using 2.5% hydrogen peroxide (LabChem, Zelienople, USA), and the mesial surface of the molars was additionally disinfected with 2.5% sodium hypochlorite (Clinix, London, UK). Cavities were prepared on the mesial surfaces of the first upper molars in all 8 rats, and the pulps were perforated with the aid of a No.1/2 round bur (Meisinger, Neuss, Germany). Working length (WL), i.e., the size of the exposed region, was equal to the diameter of the bur (0.6 mm), determined with paper points. The exposed region underwent a thorough rinsing procedure utilizing a solution comprising 0.5% sodium hypochlorite (Clinix) and 15% ethylenediaminetetraacetic acid (EDTA; Saver, Tekirdag, Turkey). In the study group, peptide amphiphiles (PAs) were applied to the dental pulp, and the cavities were sealed with a self-curing glass ionomer cement filling material (Riva Self Cure; SDI Ltd., Bayswater, Australia). In the control group, physiological saline (Deva, Istanbul, Turkey) was injected into the created defect area. Thirty days afer application, the rats were euthanized by employing a solution of 3% paraformaldehyde and 0.2%

glutaraldehyde. The teeth were subsequently extracted through surgical means and immersed in a 3% paraformaldehyde solution for 24 h. Subsequently, the tooth samples underwent demineralization using 10% EDTA and were sectioned with a Leica microtome (Leica Camera AG, Wetzlar, Germany) into 5-µm thick slices. The sections underwent deparaffinization using xylene, followed by rehydration through a sequence of ethanol solutions. Finally, the sections were stained with H&E. The slides were analyzed using the ImageJ software (National Institutes of Health (NIH), Bethesda, USA) at ×5 objective magnification to calculate the percentage of pink area in defined sites for each sample.

For Masson's trichrome staining, paraffin-embedded sections on slides were fixed in Bouin's solution, a fixative commonly used to preserve tissue morphology. The slides were then subjected to a series of staining steps to visualize specific tissue components. Initially, the slides were incubated in Weigert's iron hematoxylin solution, which selectively stained the nuclei of cells in a dark black color. Subsequently, the slides were exposed to Biebrich scarlet-acid fuchsin, resulting in the staining of collagen fibers in a vibrant red hue. Finally, aniline blue was applied to the slides, causing the collagen fibers to appear as a distinct blue color. Throughout the staining process, extensive washing steps were performed to ensure proper removal of excess dye and optimization of staining specificity. This staining protocol enabled the clear differentiation of collagen fibers (blue) from the cell nuclei (black). In addition to H&E staining, Masson's staining was performed to assess the presence of regenerative tissue sections within the pulp samples, providing valuable insights into the regenerative capacity of the treated tissues.

Statistical analyses

Statistical analyses were performed using IBM SPSS v. 24.0 (IBM Corp., Armonk, USA). The percentages of regeneration and mean color intensity of regenerated areas in rats of the study and control groups were analyzed statistically using the Mann–Whitney U test. A p-value of less than 0.05 was deemed to be statistically significant.

Results

In this study, GAG-PA and K-PA molecules were synthesized through solid-phase peptide synthesis to mimic sulfated GAGs and induce nanofiber formation. The GAG-PA was designed with functional groups such as sulfonate, hydroxyl and carboxylate to mimic GAGs. The K-PA, a positively charged molecule, was combined with GAG-PA to facilitate nanofiber formation through electrostatic interactions. The peptide amphiphiles that were synthesized underwent characterization through LC–MS analysis and subsequent purification using preparative HPLC. Scanning electron microscopy images revealed the formation of porous nanofiber networks when GAG-PA and K-PA were mixed. Circular dichroism spectrum analysis indicated a predominant β-sheet secondary structure in the self-assembled peptide amphiphile nanofibers. To assess the regeneration of tissue sections in the damaged pulp, H&E staining and Masson's trichrome staining were performed. The degree of tissue regeneration in the affected area was evaluated using these staining techniques (Fig. 3).

We carefully adhered to the requirement of providing the average baseline characteristics of the animals (e.g., age, weight, gender, microbiological status) at the beginning of the experiment. By diligently documenting these parameters, we ensured the transparency and validity of our research findings. The age and weight information allowed us to assess any potential age-related or weight-related effects on the outcomes of the experiment. We also recorded the gender of the animals to account for any gender-specific variations or influences. Furthermore, including the microbiological status provided crucial insights into the overall health and potential microbial factors that might impact the experimental results. All in all, our study respected and met the essential guideline of providing the average baseline characteristics of the animals, thereby contributing to the rigor and comprehensiveness of our research.

After 30 days, the implanted region demonstrated tissue regeneration that resembled pulp, characterized by the absence of noticeable neutrophil infiltration. The regeneration percentage in the damaged area was evaluated using H&E staining (Fig. 4). Additionally, Masson's trichrome staining confirmed the formation of reparative dentin (Fig. 5). These observations were made in both the study and control groups at the 30-day mark. However, statistical analysis revealed no significant difference between the 2 groups ($p > 0.05$). The statistical values and plots for the regenerated area and mean color intensity are provided in Table 1 and Fig. 6. Figure 6 presents the plots for the regenerated area and mean color intensity. Plot A displays the bar plots representing the mean regenerated area (%) for the serum and gel groups, with error bars indicating the standard deviation (SD) of the mean. In Plot B, a box-and-whisker plot shows the distribution of the regenerated area (%) for the serum and gel groups. For the serum group, the 1st quartile (Q1), the median and the 3rd quartile (Q3) were 85.86%. For the gel group, Q1 was 81.77%, the median was 92.10% and Q3 was 92.29%. Plot C features bar plots representing the mean color intensity (%) for the serum and gel groups, with error bars indicating the SD of the mean. Similarly, Plot D shows a box-and-whisker plot for the distribution of mean color intensity (%) for the serum and gel groups. For the serum group, Q1, the median and Q3 were 45.06%. For the gel group, Q1, the median, and Q3 were 61.42%, 68.04% and 74.65%, respectively.

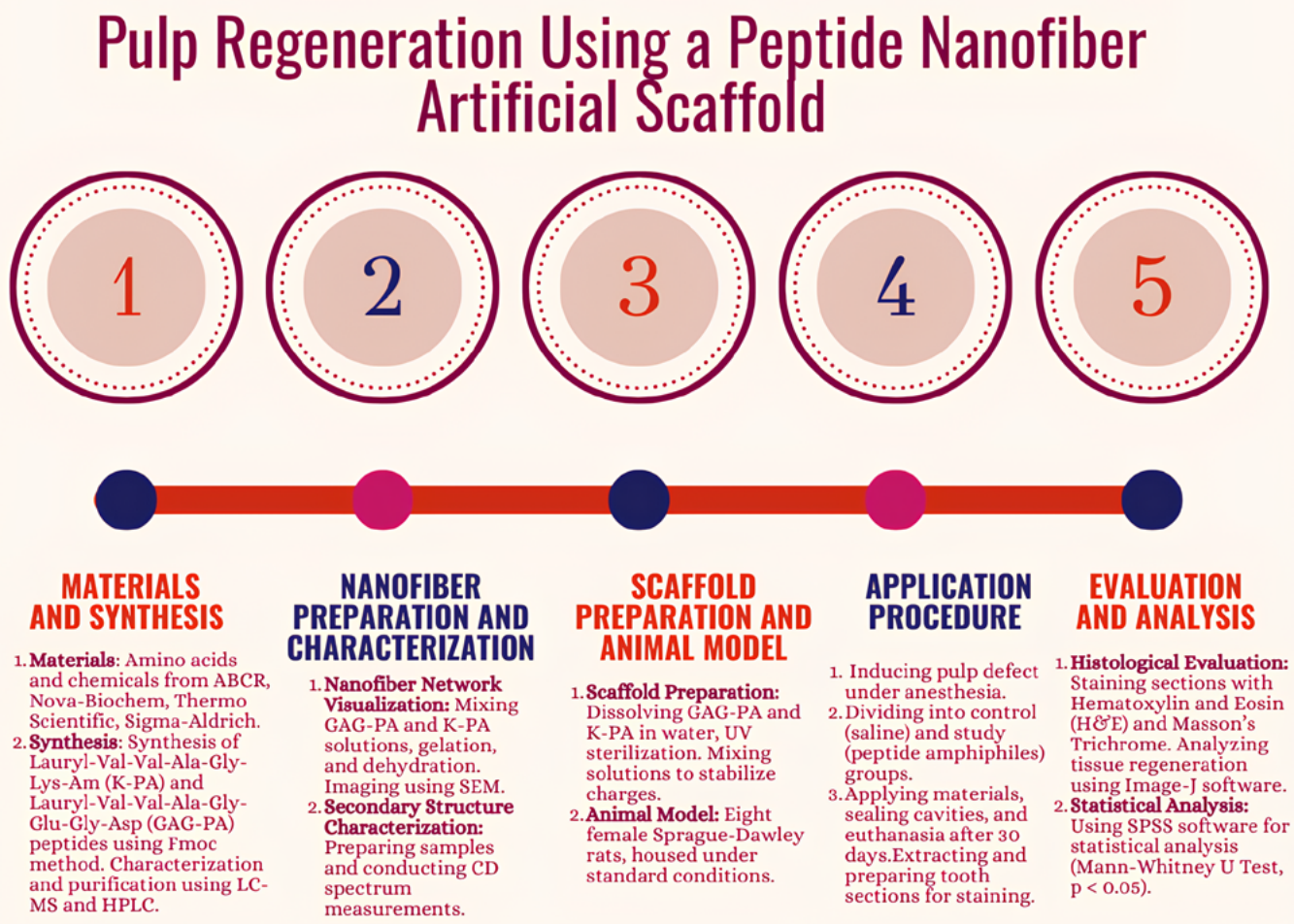

Fig. 3. Diagram of tissue regeneration evaluation after 30 days

GAG-PA – glycosaminoglycan mimetic peptide amphiphile; K-PA – positively charged peptide amphiphile; LC-MS – liquid chromatography–mass spectrometry; HPLC – high-performance liquid chromatography; SEM – scanning electron microscope; CD – circular dichroism; H&E – hematoxylin and eosin.

Discussion

In this research, dynamic nanostructures were produced by utilizing hydrogen bonding between peptide sequences and utilized in the field of dental pulp tissue engineering for pulpotomized rat molars. Over 30 days, the previously pulpotomized area of the pulp was successfully replenished with regenerated tissue, demonstrating an absence of inflammation. These findings strongly suggest that hydrogel scaffolds incorporating GAG-PA nanofibers play a significant role in promoting dental pulp regeneration.

In the present study, PA nanofibers were injected into the pulp tissue of rats, and regenerated tissue was observed in the pulpotomized region. Cordeiro et al.[33] conducted a study wherein dental pulp stem cells were seeded onto poly L-lactide acid (PLLA) scaffolds positioned within the pulp-chamber space of human tooth slices, which were subsequently implanted subcutaneously in mice. The resulting tissue exhibited a comparable architecture and cellularity to dental pulp tissue, although it did not fill the entire pulp space. Ito et al.[7] presented a protocol for in vivo pulp tissue engineering in pulpotomized rat teeth involving rat bone marrow mesenchymal stem cells (RBMMSCs), preformed biodegradable scaffolds and hydrogels. Consistent with our findings, the implantation of RBMMSCs, along with preformed scaffolds and hydrogels, resulted in pulp tissue regeneration within pulpotomized pulp chambers in rats.

Previous studies have shown that nanostructures consisting of certain peptides can be used for cell therapy.[34–38] For example, when the RGDS signal in fibronectin binds cells in a network formed by nanostructures, the cells can live in the artificial matrix.[39,40] This study was conducted in collaboration with the Ulusal Nanoteknoloji Araştırma Merkezi (UNAM)-National Nanotechnology Research Center (Ankara, Turkey), which synthesizes these nanostructures.

Inflammation of dental pulp tissue with the progression of dental caries causes severe pain, which can be relieved by root canal treatment. Teeth that have undergone root canal treatment cannot renew themselves, and their life is shortened. Therefore, problems related to the dental pulp are important for endodontists and pedodontists. Pulp regeneration to revive dentine-producing odontoblasts and produce new capillaries and nerve cells can be an effective alternative method for endodontic therapies.

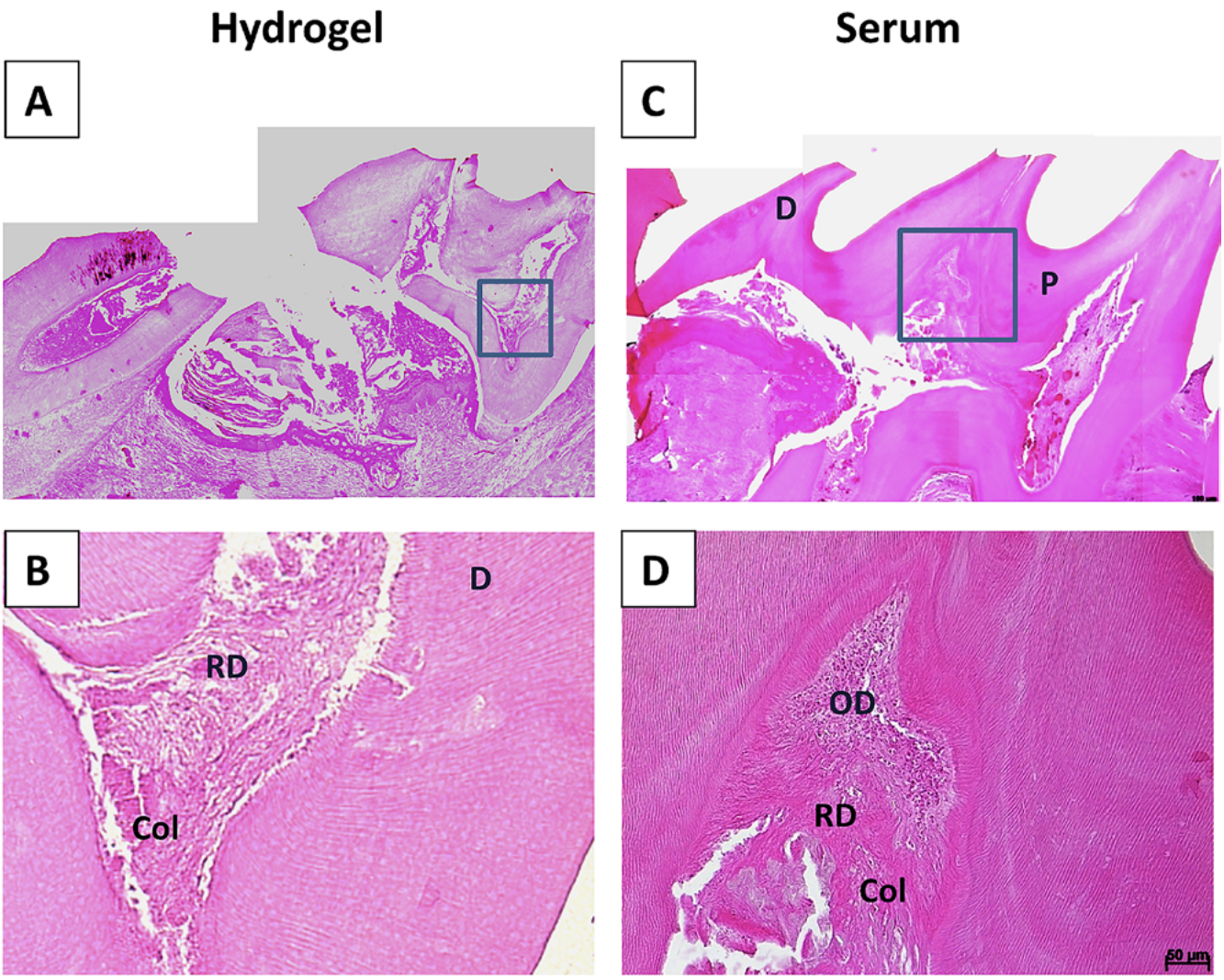

Fig. 4. Hematoxylin and eosin (H&E) staining. Hydrogel/serum was injected into the rat's molar tooth, and the rat was sacrificed after 4 weeks of injection. A,B. Hydrogel injection; C,D. Serum injection

D – dentin; P – pulp; DS – defect site; Col – collagen; RD – reparative dentin; OD – odontoblasts.

Within the scope of tissue engineering studies, dental pulp tissue production is of great importance in REP. We believe that the bioactive intercellular scaffold consisting of peptides generated in this study, when applied together with growth factors, may induce dental tissue formation in the region where it is applied.

In the literature, favorable outcomes of regenerative treatment procedures are frequently discussed. However, a systematic review conducted in 2022 revealed a lack of robust evidence supporting the effectiveness of regeneration procedures in necrotic immature teeth.[17] The development of a healthy dentin-pulp complex is not solely dependent on the survival of stem cells from the apical papilla (SCAPs). It also requires the presence of epithelial cells derived from Hertwig's epithelial root sheath (HERS) and their interaction with epithelial rests of Malassez (ERM). Hertwig's epithelial root sheath functions as a barrier between dental follicles and dental papilla cells, and it has regulatory properties that determine the shape, number and dimensions of roots.[6,41] Normal and healthy development of the dentin-pulp complex necessitates the survival of HERS, ERM and SCAP. Therefore, defining regeneration procedures is crucial. Additionally, in human and animal studies targeting endodontic regeneration, it is accepted that internal periodontal tissues regenerate more significantly than pulp tissue formation in root canals. This is attributed to the formation of bone-like tissue, periodontal ligament and cementum in place of dentin walls, as reported in histological examinations.[6,24]

To improve the effectiveness of biomaterials and regenerative drugs in cell therapy, it is necessary to support the vital activities of the cells in the early stages, and the biomaterial should later disappear through biological destruction, preventing the development of natural tissue.[42] The matrix consists of synthetic biomaterials and provides mechanical support to the cells and can carry proteins, biological signals, nutrients, and various genes, which increases its utility. In previous studies,

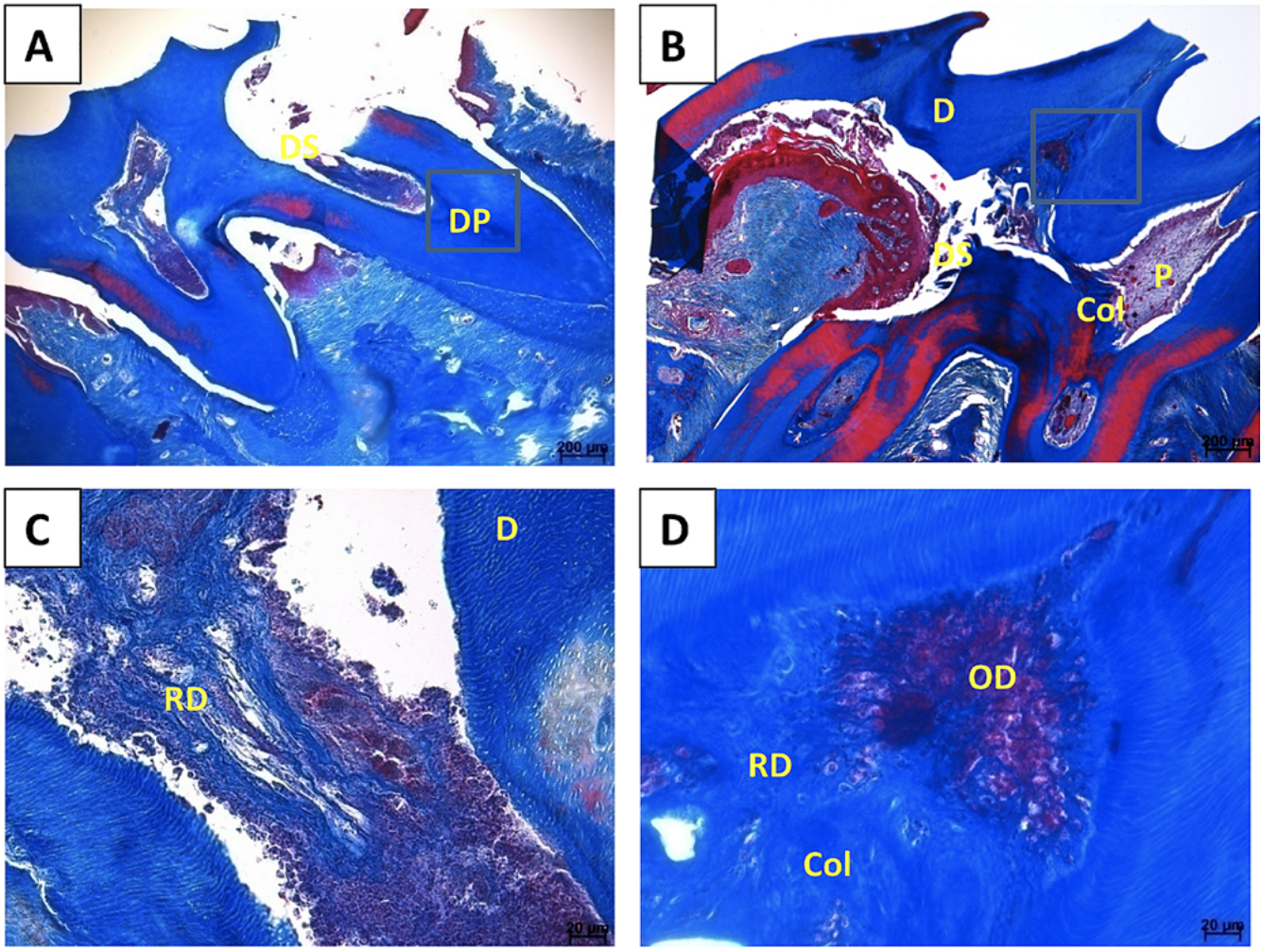

Fig. 5. Masson's trichrome staining. Hydrogel/serum was injected into the rat's molar tooth, and the rat was sacrificed after 4 weeks of injection. A,C. Collagen deposition and reparative dentin formation in the hydrogel group; B,D. Collagen organization and odontoblast alignment in the serum group

D – dentin; P – pulp; DP – damaged pulp; DS – defect site; Col – collagen; RD – reparative dentin; OD – odontoblasts.

the development of multipurpose biomaterials with some programmable collectible molecules made it possible for cells to survive outside their natural environment in bioactive matrices consisting of dynamic, natural and chemical intermediates.[43] Intense efforts are being made in the field of tissue engineering and regenerative medicine to develop materials that contain peptide signals and can form complex supramolecular nanostructures. Bioactive peptide sequences can be used to mimic the natural cell environment in the matrices of such molecules.[44]

Based on the results of our study, although no difference was observed between the control and study groups, we believe a significant difference could be observed with an increase in the number of samples. In addition, the regeneration without any inflammation in the samples in the study group was considered a success.

The biomechanical properties of dental pulp and scaffolds are integral considerations in advancing regenerative dentistry and dental tissue engineering. Dental pulp, situated within the pulp chamber, exhibits essential characteristics such as compressive strength, crucial for withstanding occlusal forces during biting and chewing, and elasticity, which enables it to act as a shock absorber, protecting the underlying tissues.[45] The vasculature within dental pulp contributes significantly to tissue vitality and regenerative potential. In parallel, the biomechanical properties of scaffolds play a pivotal role in dental tissue engineering. Scaffolds must offer structural integrity to support tissue regeneration, be biocompatible to facilitate cell interactions, and possess controlled degradation characteristics to align with the pace of tissue healing.[46] Additionally, the design of scaffolds should include appropriate porosity and permeability to allow for nutrient and oxygen diffusion, fostering cell ingrowth. Achieving a biomechanical match between scaffolds and native dental tissues is paramount for successful outcomes in regenerative dentistry applications, ensuring optimal support for tissue regeneration and functional restoration.[47]

The materials developed in this study can be examined for therapeutic potential in humans. The injectable structure of the scaffold may be used for treating dental problems. Thus, further studies are needed for the development

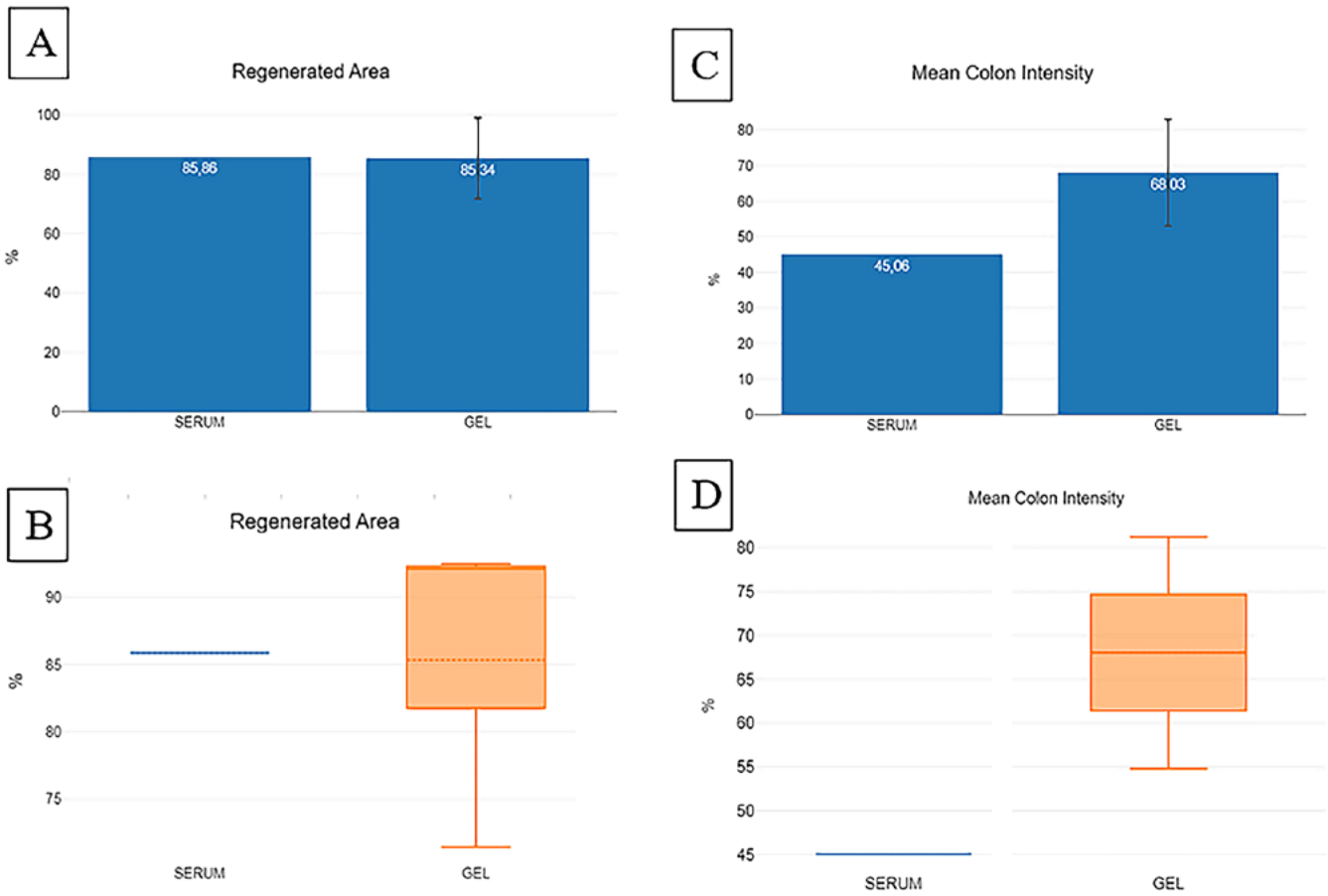

Fig. 6. Plots for the regenerated area and mean color intensity. The box represents the interquartile range (Q1 to Q3), the line inside the box indicates the median, and the whiskers extend to the minimum and maximum values excluding outliers. A. Bar plot representing the mean regenerated area (%) for serum and gel groups, with error bars indicating standard deviation (SD); B. Box-and-whisker plot showing the distribution of regenerated area (%) for serum and gel groups; C. Bar plot for mean color intensity (%) with error bars indicating SD; D. Box-and-whisker plot depicting the distribution of mean color intensity (%) for serum and gel groups, detailing interquartile range (IQR), median, and outlier data points

and production of biomaterials that have the characteristics of controlled aggregation, the transmission of biological signals to cells, imitation of the natural cell environment, and synergistic effects with biological factors.

Several studies have emphasized the benefits of using animal models in REP. Animal models allow for controlled experiments, providing insights into biological processes and potential therapeutic outcomes. For example, a study by Nakashima et al.[43] highlighted the ability of an animal model to accurately simulate pulpal regeneration and evaluate the effectiveness of novel biomaterials. However, it is crucial to acknowledge the limitations associated with animal models. Variability in anatomy, physiology and immune responses can exist between animals and humans, affecting the translatability of findings. Previous studies, such as Piglionico et al.,[44] have emphasized the importance of considering these differences when interpreting results from animal studies and applying them to human REP.

Dental pulp regeneration is a dynamic process involving the proliferation and differentiation of various cell types, along with the deposition of extracellular matrix components such as dentin. The 30-day experimental duration captured critical early events in the regenerative process, including the inflammatory response, cell migration and initial matrix deposition.[45] This timeframe strikes a balance between obtaining meaningful data on the effectiveness of GAG-PA nanofibers in guiding pulp regeneration and minimizing potential ethical concerns related to prolonged experimental periods for laboratory animals. While the study successfully observed reparative dentin formation, the acknowledgment of a lack of statistically significant differences between the study and control groups suggests that a longer observation period or an increased number of animals may be necessary to capture more advanced stages of regeneration and detect significant treatment effects. The researchers chose a 30-day duration as an initial exploration, recognizing the potential for further investigations with refined methodologies and extended timelines to elucidate the full scope of the regenerative outcomes.

In this study, rat teeth were used due to their similarities to human teeth in terms of size, structure and biological properties. These similarities make rat teeth a valuable model for studying the efficacy and safety of biomaterials in REP. Also, rats are commonly used in scientific research due to their abundance, ease of breeding and short reproductive cycle. Additionally, ethical considerations

come into play, as using rats as an alternative to human teeth helps minimize potential harm and discomfort to human subjects. Conducting experiments on rat teeth is relatively cost-effective and practical compared to using human teeth. Rat teeth can be obtained at a lower cost and in larger quantities, allowing for more extensive experimentation and analysis. Ethical guidelines and regulations often require initial testing on animals before moving to human trials. By using rat teeth, researchers can comply with these regulations and gather essential preliminary data before considering human clinical trials.

To apply the results of this manuscript to humans, further studies should be conducted on larger animal models that more closely resemble human dental anatomy and physiology. While the current study utilized rats, which are a common choice for initial investigations due to their ease of handling and cost-effectiveness, translating these findings to the human context requires studies on larger animals with dental structures more akin to humans. Animal models such as non-human primates are often preferred for dental research because they share similarities in tooth structure, size and dental pulp characteristics with humans. Conducting studies on these larger animals would provide a more representative evaluation of the effectiveness of GAG-PA nanofibers in guiding pulp regeneration, helping to bridge the gap between preclinical experiments and potential human applications. Additionally, increasing the sample size and diversity in animal models can contribute to a more robust statistical analysis, addressing the observed limitations in the current study and enhancing the reliability of the results for potential clinical relevance in humans.

The clinical or radiographic outcomes of REP remain somewhat unpredictable. However, in vivo studies of scaffolds lead to clinical studies.[20,25,41] The findings of our study will inform future research, which will aim to identify the relative efficacy of different scaffolds. Various materials that will provide different functionalities to scaffolds continue to be produced. Examples include antimicrobial peptides (AMPs) such as LL37 peptides and various nanoscale scaffolds.[46,48] However, AMPs have limited therapeutic effects due to their short residence time in the circulatory system and their sensitivity to proteases. These limitations are major obstacles to the success of AMPs.[46,47] In contrast, nanoscale scaffolds, hydrogels and various scaffolds can be added to AMP-based materials to increase the therapeutic efficacy of the material.[48] It is possible that nanoscale scaffolds and antimicrobial proteins such as LL37 peptides may explain their activity on regeneration. Further pioneering studies similar to our study are needed to elucidate this issue.

Limitations

Our study encountered certain limitations, including the utilization of rat teeth instead of human teeth and the relatively small sample size. Animal models serve as indispensable tools for researchers to attain a comprehensive understanding of diseases, advance the development of effective treatments and explore innovative ideas, concepts and technologies. These animal models play a vital role in conferring scientific validity to their investigations. When evaluating various therapeutic approaches in the domain of REP, reliable animal models simulating pulpal defects assume significant importance. While the structural and compositional differences between rat incisors and human teeth are considerable, the similarities in structural characteristics, such as the pulp chamber, pulp tissue, root, and apical delta with minor apical foramen, make rat and human molars more comparable. Furthermore, the use of rats in REP research proves advantageous due to the lower costs and efforts associated with housing, feeding, and care compared to larger animals. Additionally, most of the antibodies necessary for cellular and molecular biological techniques are specifically available for rats.

Conclusions

In this study, reparative dentin formation was observed in both groups, but without a significant difference between the groups. To obtain a significant difference, the number of animals used must be increased. Moreover, the PA-based nanomaterials used in this study were nontoxic to the dental tissue and were digested by the enzyme in dental tissue over time. Bioactive signals were presented to the sensing proteins effectively and functionally using the wide reaction surface features of the nanostructures. Our findings indicate that these biomaterials can be used to produce molecules for application in the regeneration of dental tissue and dental pulp, thus leading to improved quality of human life.

Data availability

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

Consent for publication

Not applicable.

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