https://doi.org/10.31925/farmacia.2024.6.17 **ORIGINAL ARTICLE**

IN VITRO AND *IN OVO* COMPARATIVE TOXICOLOGICAL ASSESSMENT OF SELF-ASSEMBLING PEPTIDE-BASED DENTAL REPAIR AGENTS

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Manuscript received: August 2024

Abstract

A significant health issue is that dental demineralisation can cause dental caries and other systemic health concerns, negatively impacting overall well-being. Due to the developmental stages of enamel and dentin in children, they are particularly vulnerable. This study aimed to evaluate the safety of two self-assembling peptide-based gels (SAPs) used for dentin remineralisation. For the evaluation of SAPs', they were tested *in vitro* on human gingival fibroblasts (HGF) and *in ovo* on the chorioallantoic membrane of hen's eggs. Viability, morphology and nuclear integrity were evaluated *in vitro*, while irritation potential was determined *in ovo*. As a result of the findings, SAPs do not present a significant degree of cytotoxicity, providing minimal irritation and a protective, anti-irritating effect. Therefore, SAP-based gels suggest a biocompatible, safe and effective method for remineralisation, with low cytotoxicity and minimal irritation risk.

Rezumat

O problemă majoră de sănătate, demineralizarea dentară, poate provoca carii dentare, precum și alte probleme sistemice de sănătate, cu impact negativ asupra bunăstării generale. Datorită stadiilor de dezvoltare ale smalțului și dentinei la copii, aceștia sunt vulnerabili. Scopul studiului a fost evaluarea siguranței a două geluri pe bază de peptide cu auto-asamblare (SAP) utilizate pentru remineralizarea dentinei. Pentru evaluarea SAP-urilor, acestea au fost testate *in vitro* pe fibroblaste gingivale umane (HGF) și *in ovo* pe membrana corioalantoidiană a ouălor de găină. Viabilitatea, morfologia și integritatea nucleară au fost evaluate *in vitro*, iar potențialul de iritare a fost determinat *in ovo*. Conform constatărilor, SAP-urile nu prezintă un grad semnificativ de citotoxicitate, având potențial iritativ minim și un efect protector, anti-iritant. Astfel, gelurile pe bază de SAP sugerează o metodă biocompatibilă, sigură și eficientă de remineralizare, cu citotoxicitate scăzută și risc minim de iritare.

Keywords: dentin demineralisation, self-assembling peptide-based gels, human gingival fibroblasts, chorioallantoic membrane

Introduction

Dental demineralisation disorders are among the most important global health concerns; statistics indicate that almost all people on the planet suffer from these medical conditions at some time in their lives. The paediatric population and the field of pedodontics are

worth mentioning, considering that children are more vulnerable to the appearance and development of dental caries [3]. In terms of clinical manifestations, aesthetics and quality of life of patients, the loss of mineralised tissue has a significant impact [17]. It is important to note that dentin demineralisation poses a complex issue affecting the structure and function of the mineralising tissues. Several factors contribute to the onset of these conditions, including mechanical wear, abrasion, biofilm infections and abnormalities of homeostasis and metabolism, among others [17, 25, 34]. The main risk associated with the dentine demineralisation is that it may affect the integrity of the teeth, which may lead to the development of dental caries. This process is mainly mediated by acid-producing bacteria that are involved in the metabolism of fermentable carbohydrates and that contribute to the dissolution of hydroxyapatite crystals [42]. Furthermore, a decrease in mineral content leads to an increase in the sensitivity of the teeth, particularly to mechanical and thermal stimuli, which results in pain [16]. To effectively manage dentine demineralisation, it is necessary to consider the interplay between diet, oral hygiene and salivary composition [21].

Treatment for dentine demineralisation involves both pharmacological and non-pharmacological measures. To maintain the integrity of the dentin, it is imperative to change the lifestyle and diet, reducing consumption of carbohydrates and other foods that damage the dentin [37]. Performing periodic dental examinations is also crucial for the detection of dental problems at an early stage and the prevention of dental disorders by professional cleaning methods [20]. Conversely, fluoride-based treatments are highly effective in remineralising teeth, both through the creation of mineral deposits within the dentine matrix and through the inhibition of cariogenic bacteria [31].

The use of fluoride therapy, however, is controversial due to the possibility of causing enamel discolouration and staining, and long-term exposure could affect neuronal development and lead to skeletal fluorosis [1, 18, 46]. Thus, new therapeutic approaches to dentine demineralisation were sought. One fairly common approach is the use of casein phosphopeptide-amorphous calcium phosphate, which has proven to be highly effective, but can cause allergic reactions in individuals who are lactose intolerant [36]. In addition, bioactive glass, although effective, requires special attention when applying as it can cause discomfort or sensitivity, and its effectiveness depends on the composition of the saliva [35].

Dental repair agents based on self-assembling peptides (SAP) are an innovative method of treating dentine demineralisation. A fundamental principle of these peptides is the formation of spontaneous nanostructures under physiological conditions, mimicking the extra-cellular matrix and stimulating the regeneration of dental tissues. In order to promote the natural healing process, the SAPs attract calcium and phosphate ions at the level of damaged enamel [33]. Among the advantages of these agents is that they enhance the tooth's structural integrity and limit the need for invasive procedures such as fillings [43]. Even though SAPs are highly effective, and peptides are generally considered biocompatible due to their biomimetic nature, more studies are required to determine their safety and cytotoxicity [30].

Accordingly, this study aimed to test two gels based on self-assembling peptides (SAP1 and SAP2) *in vitro* and *in ovo*. The purpose of this article was to determine the cytotoxic potential *in vitro* by applying methods for determining cell viability and evaluating the morphology of the cells and the nuclear structure. Furthermore, the products were tested *in ovo* for their irritating and anti-irritating effects on the chorioallantoic membrane.

Materials and Methods

Reagents

Sodium chloride was purchased from Chimopar S.A. (Bucharest, Romania). Potassium chloride was obtained from Chimreactiv (Bucharest, Romania). Calcium chloride dehydrate was delivered by Honeywell Fluka™ (Charlotte, NC, USA). Urea and the MTT Cell Viability Kit were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fibroblast Basal Medium (PCS-201), Fibroblast Growth Kit - Low Serum (PCS-201-04), penicillin/streptomycin/amphotericin B (PCS-999-002™) were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA. The Hoechst 33342 was provided by ThermoFisher Scientific (Waltham, MA, USA).

Equipment

The equipment used in this study (Eutech pH 150 electrode pH meter, Cytation 5 plate reader, Lionheart FX automated microscope, Gen5™ microplate data collection and analysis software version 3.14, and SteREO Discovery. V8 stereomicroscope) were provided by BioTek Instruments Inc. (Winooski, VT, USA), ZEISS (Jena, Germany) and Thermo Scientific, Waltham, MA, USA.

Artificial Saliva and Sample Preparation

The artificial saliva was prepared by dissolving 0.40 mg/L NaCl, 0.40 mg/L KCl, 0.80 mg/L $CaCl_2 \cdot 2H_2O$ and 1 mg/L urea in distilled water, according to a previous publication [13]. The pH was determined with an Eutech pH of 150 [12]. The formulation of the artificial saliva used in the present study was chosen to mimic saliva's composition and natural function as closely as possible since it plays a fundamental role in dentine demineralisation. Calcium, phosphate and sodium ions were used in saliva's composition to reproduce saliva's remineralisation capacity and pH stabilisation agents. The formulation was designed to provide an environment as similar as possible to the physiological one for a more appropriate evaluation of the gels. The formulation

also maintains a physiological pH range (typically 6.5 to 7.5), essential for safeguarding enamel integrity and promoting the natural defence mechanisms of the oral environment. By replicating the ion concentrations and pH levels of natural saliva, the artificial saliva provides a realistic environment that supports its protective and restorative roles, enabling reliable evaluation of dental treatments. The two gels tested in this study are commercially available products specifically designed for dental use. They were prepared and supplied directly by their respective manufacturers, ensuring consistent composition and quality. Both gels contain self-assembling peptides (SAPs) as active components to support dentin remineralisation and improve oral health. The samples were immersed in artificial saliva (1 mg sample/1 mL artificial saliva) at neutral pH for seven days in an incubator with an orbital shaker (ES20/60 Biosan, Riga, Latvia) at a constant temperature of 37°C and further diluted in water or culture medium at a dilution of 1:16, 1:8, 1:4, 1:2 and 1:1, respectively. The dilutions used in the experiments (1:16, 1:8, 1:4, 1:2, 1:1) were selected based on a previous study that evaluated the bio-compatibility and biosafety of dental materials immersed in artificial saliva within the same dilution range on HaCaT cells [13]. Physiological relevance was balanced with the need to evaluate concentration-dependent effects in the 1:16 to 1:1 dilution range. Across this range, the SAP gels can be tested under varying levels of exposure, from highly diluted conditions to undiluted or minimally diluted states, to mimic natural saliva interactions. Dilution of 1:16 simulates the gradual dispersion and mixing of the gel with saliva in the oral cavity, enabling an evaluation of the gel's effectiveness in routine use. In addition, the 1:1 dilution is used to determine the safety and efficacy of the formulation when applied directly, ensuring no adverse effects. Through testing across this spectrum, the gels' biocompatibility and functional performance can be assessed, as well as variations in cell viability, irritation potential and therapeutic effectiveness under different conditions of use.

Cell Culture Conditions

For *in vitro* experiments, the human gingival fibroblast cell line HGF (PCS-201-018™) was grown in specific fibroblast growth medium supplemented with Fibroblast Growth Kit-low serum and penicillin (10 units/mL) – streptomycin (10 µg/mL) – amphotericin B (25 ng/mL) (PCS-999-002™) under specific conditions, in an atmosphere of 5% CO_2 and a temperature of 37°C during the experiment.

Cellular Viability Assessment – MTT

To determine the effect of the samples (at dilutions 1:16, 1:8, 1:4, 1:2 and 1:1) on cell viability, the HGF cells were cultured in 96-well plates (10^4 cells/well) and stimulated for 72 hours. After this time, the culture medium was replaced with fresh medium, then 10 µL/well of kit-1 MTT was added, and the plate was left to incubate for three hours (at 5% CO_2 and 37°C). Subsequently, 100 µL/well of kit-2 MTT was added, the plates were incubated at room temperature for 30 min, and the absorbance was read at 570 and 630 nm wavelengths using Cytation 5.

Cellular Morphology

The cellular morphology of HGF cells was determined after 72 h of treatment using an Olympus IX73 inverted microscope. The cells were grown and incubated under standard conditions (5% CO_2 and a temperature of 37°C) in 12-well plates (1 x 10^5 cells/well) and treated with the samples of interest when the desired confluence was reached. The photographs were taken at magnification 20x, exposure time 10 - 50 ms and resolution 2048 x 2048 pixels, and analysed using cellSens Dimensions v.1.8 Software/Gen5 v3.14. Software.

Nuclear Morphology- Hoechst Staining

The Hoechst 33342 staining assay reveals possible cytotoxic effects on the nuclei of HGF cells that may appear after 72 hours of treatment. After this period, the culture medium was replaced with 500 µL/well of Hoechst solution (1:2000 in PBS), and then the plate was incubated in the dark for 5 - 10 min. Finally, the wells were washed with PBS, and photographs were taken at 20 x magnification, exposure time 100 ms, resolution 2048 x 2048 pixels, under a DAPI filter, with a Lionheart FX automated microscope, which was then analysed using Gen5 microplate Data Collection and Analysis Software.

To calculate the apoptotic index (AI), the following formula was applied [39]:

$$AI = \frac{\text{Number of apoptotic cells}}{Total \text{ number of cells}} x100.$$

Hen's Egg Test on Chorioallantoic Membrane (HET-CAM) Assay

The HET-CAM method used white fertilised chicken eggs to monitor and quantify the anti-irritant potential of SAP1 and SAP2. On the first day, the eggs were placed and maintained in an incubator for three days under standard conditions (37°C and 60% humidity). On the fourth day, 6 - 7 mL of egg white was removed with a syringe needle through a small cut in the top of the egg, which was then covered with adhesive tape. The following day, a small window was cut on each egg so that the chorioallantoic membrane could be seen more clearly, and this opening was covered with adhesive tape [26].

The experiment was performed on the tenth day of incubation by applying 600 µL of each sample. A positive control consisting of 1% sodium dodecyl sulphate (SDS) and a negative control (purified distilled water) were tested in parallel. Possible changes such as haemorrhage (H), lysis (L) and vascular coagulation (C) were monitored for 5 min by comparing a photograph of the membrane taken before sample application (T0) with a picture taken 5 min after sample application (T5) using the Discovery v.8 stereomicroscope and the ZEN core 3.8 software [14].

The irritant potential of the samples was determined by calculating the irritation score (IS) using the following formula:

$$IS = 5x\,\frac{301-H}{300} + 7x\,\frac{301-L}{300} + 9x\,\frac{301-C}{300}.$$

The samples were classified according to the results obtained into non-irritating, irritating and severely irritating, according to the specific ranges in the literature [9].

The method previously described in the literature was applied to determine the anti-irritant potential [28]. Thus, SAP1 and SAP2, previously tested by the HET-CAM method, were applied in a volume of 300 µL, and the eggs were incubated for one hour for the solution to be absorbed entirely. After this time interval, 300 µL of 1% SDS solution was added to chorioallantoic membrane, and the vascular changes (haemorrhage, lysis and coagulation) were observed and analysed similarly to the previously described HET-CAM method. Finally, the following parameters were calculated:

$H_{AI} = H/H_{SDS}$, haemorrhage time after pre-treatment with SAP1 and SAP2 and 1% SDS addition *per* haemorrhage time without pre-treatment;

$L_{AI} = L/L_{SDS}$, vascular lysis time after pre-treatment with SAP1 and SAP2 and 1% SDS addition *per* vascular lysis time without pre-treatment;

$C_{AI} = C/C_{SDS}$, vascular coagulation time after pre-treatment with SAP1 and SAP2 and 1% SDS addition *per* vascular coagulation time without pre-treatment.

Statistical Analysis

Statistical interpretation was performed with GraphPad Prism version 9.4.0 for Windows, GraphPad Software, San Diego, CA, USA. The results were expressed as mean ± standard deviation (SD) and analysed by one-way ANOVA statistical test, followed by Dunett's post-test. Statistical differences were marked with * (*p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001) [4].

Results and Discussion

The process of dental demineralisation involves the loss of minerals and is considered the beginning of the development of dental caries [40]. It is a serious health problem that can lead to dental decay, infections and even tooth loss if left untreated. Besides affecting oral health, these effects have a systemic impact, which affects various body functions, as well as patient well-being [29]. One innovative dental demineralisation approach is using self-assembling peptides as dental repair agents. During mineralisation, these repair agents form nanostructures that resemble natural mineralisation processes, thus promoting calcium and phosphate deposition. With their more precise mechanism of action and higher biocompatibility, these peptides represent an important alternative to fluoride-based treatments [2].

It is extremely important to evaluate peptide-based self-assembly dental repair agents *in vitro* in order to determine and comprehensively understand their mechanisms of action, safety and efficacy [41]. Consequently, the present study's purpose was to evaluate two types of gel based on self-assembling peptides (SAP1 and SAP2) at the level of human gingival fibroblasts (HGF) in terms of their viability, morphology and nucleus structure.

Regarding the cytotoxic profile of the two samples on human gingival fibroblasts, as shown in Figure 1, SAP1 and SAP2 do not show signs of cytotoxicity. Thus, according to the MTT assay, cell viability decreases slightly depending on the concentration used for each of the two samples. At 1:16 and 1:8 dilutions, for both SAP1 and SAP2, the cells appear to be stimulated compared to the control, and at 1:1 dilution, SAP1 at 1:1 dilution decreases cell viability only to about 89%. In comparison, SAP2 treatment decreases cell viability to about 85% (the lowest percentage).

As a standardised environment for evaluating dental products' biological and safety profiles, artificial saliva is frequently used in *in vitro* studies to stimulate the oral environment [7]. Human gingival fibroblasts are connective tissue cells participating in oral health, periodontal biology, extracellular matrix synthesis and maintenance and gingival tissue support.

Aside from their basic functions, HGFs are also involved in healing processes, increasing collagen production and other repair molecules in response to various injuries [27].

Due to the previously mentioned, HGF cells represent a valuable model for *in vitro* testing of different dental treatments.

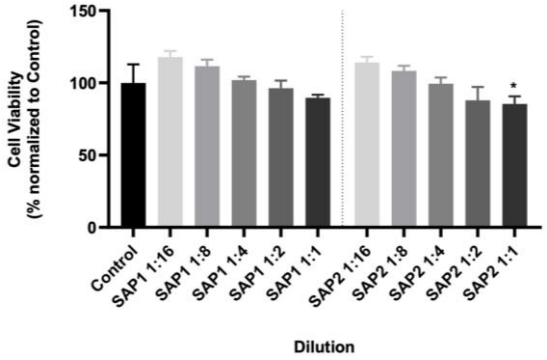

Figure 1.

Graphical representation of cell viability percentages obtained 72 h after the treatment of HGF cells with SAP1 and SAP2 (1:16, 1:8, 1:4, 1:2, 1:1 dilution) The results are presented as percentages (%), normalised to control. All data are expressed as mean values ± SD from three independent experiments performed in triplicate. For analysing the statistical differences between the control group and the treated groups, a one-way ANOVA test was conducted, followed by Dunnet's multiple comparison post-test; "*" indicates statistical significance (* p < 0.05)

These cells represent a relevant biological environment for testing the cytotoxicity and effectiveness of dental products, including adhesives, restorative materials, or compounds with an antimicrobial effect [38]. Previous research has highlighted that HGF cells can be used to evaluate the inflammatory response, making it easy to monitor the adverse effects associated with dental materials [4]. Also, HGF cells can be used to evaluate the effects of remineralisation agents, providing an overview of cellular behaviour and extracellular matrix synthesis, which is essential for periodontal health [27]. Previously, studies have used HGF cells to evaluate self-assembling peptide-based dental repair products [19]. Research has been conducted to assess the impact of these dental products that mimic the extracellular matrix, which has the ability to promote cell attachment, proliferation and differentiation [22]. Besides determining the viability of the cells, the evaluation of the cell morphology is an essential step in identifying the health status of the cells and their response to various treatments. Cell morphology provides information regarding the health of the cells and their interactions with their substrate [45]. In order to gain a better understanding of how a certain type of treatment affects the proper biological functioning of cells, it is important to combine the assessment of cell viability and cell morphology [44].

This led to the next step of the present study, which involved evaluating the morphology of HGF cells after treatment with the two types of gels.

Cell morphology was investigated 72 hours after treatment with SAP1 and SAP2 for 1:16, 1:4 and 1:1 dilution. According to Figure 2, cell morphology was not altered during the SAP1 and SAP2 treatments. At 1:16 dilution, the cells appear to be stimulated by the two samples, but at 1:1 dilution, a slight decrease in confluency can be observed.

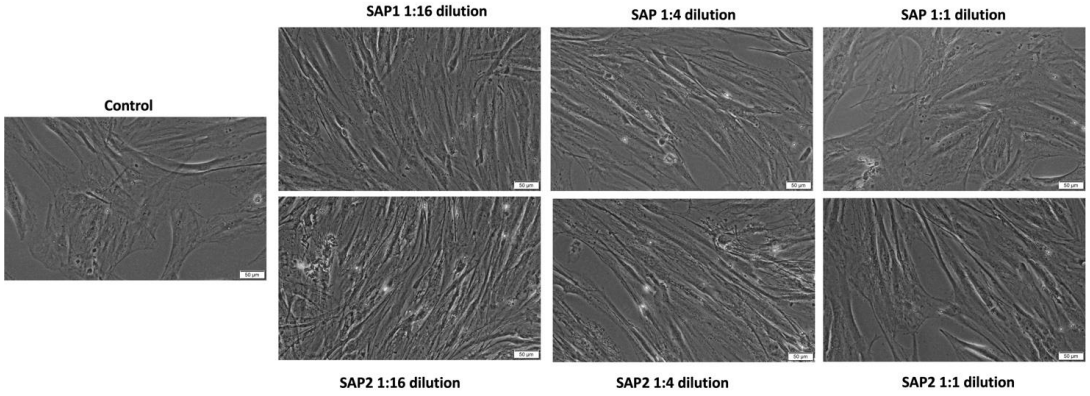

Figure 2.

Microscopic images illustrating the morphology of HGF cells were observed 72 h after treatment with SAP1 and SAP2 (1:16, 1:8, 1:4, 1:2, 1:1 dilution)

Images were captured at 20× magnification; the scale bar indicates 50 µm

Studies on the effect of self-assembling peptide-based dental repair agents on cellular morphology have been limited. Additional studies are needed better to understand their mechanism of action at the cellular level. The results of a previous study demonstrated the ability of these agents to interact with dentin and influence the binding power of collagen fibres, thereby affecting the cellular microenvironment [11]. Likewise, another study on enamel regeneration examined cellular morphological changes following treatment, confirming these agents' ability to restore enamel [2].

Evaluation of the structure of the nuclei is another important step in determining the impact of treatment at the cellular level. Hoechst staining binds to DNA in the adenine-thymine region, allowing microscopic visualisation of nuclei. This method is capable of detecting early apoptotic changes, such as chromatin condensation and the formation of apoptotic bodies [8, 10].

Hence, Hoechst staining was employed to evaluate the impact of the two products at the level of nuclei. Further, the influence of the SAP1 and SAP2 treatments was investigated after 72 h of stimulation in HGF cells. Similar to the morphological analysis, 3 dilutions were chosen for the experiment – the weakest dilution (1:16), an intermediate dilution (1:4) and the most concentrated dilution (1:1). As can be seen in Figure 3A), the changes at the nuclear level are slight, with the most noticeable changes seen in the 1:1 dilution for both SAP1 and SAP2. In the 1:1 dilution, the nuclei are condensed or rounded in places, changes marked by the white arrow. Changes at the nuclear level, such as condensation of nuclei, reduction in size and rounding, were observed during the study. These alterations are hallmarks of cellular stress or early apoptosis, indicating potential cytotoxic effects. Nuclear condensation involves the compaction of chromatin, which reduces the size of the nucleus while rounding signifies a loss of the typical nuclear morphology. These changes often reflect

the activation of cell death pathways, which may occur in response to toxic agents or unfavourable environmental conditions. In this study, the absence or minimal presence of such changes suggests that the tested gels exert low cytotoxicity, maintaining the structural integrity of the nuclei. Following the experiment, the apoptotic index was calculated (Figure 3 B), which indicated that the nuclear dysmorphologies are directly proportional to the dilution tested. Thus, the highest percentage was reached for SAP2 at 1:1 dilution (about 4.39%) compared to the control, where the apoptotic index was about 3.3%. However, no signs of marked cytotoxicity were observed for the two samples tested.

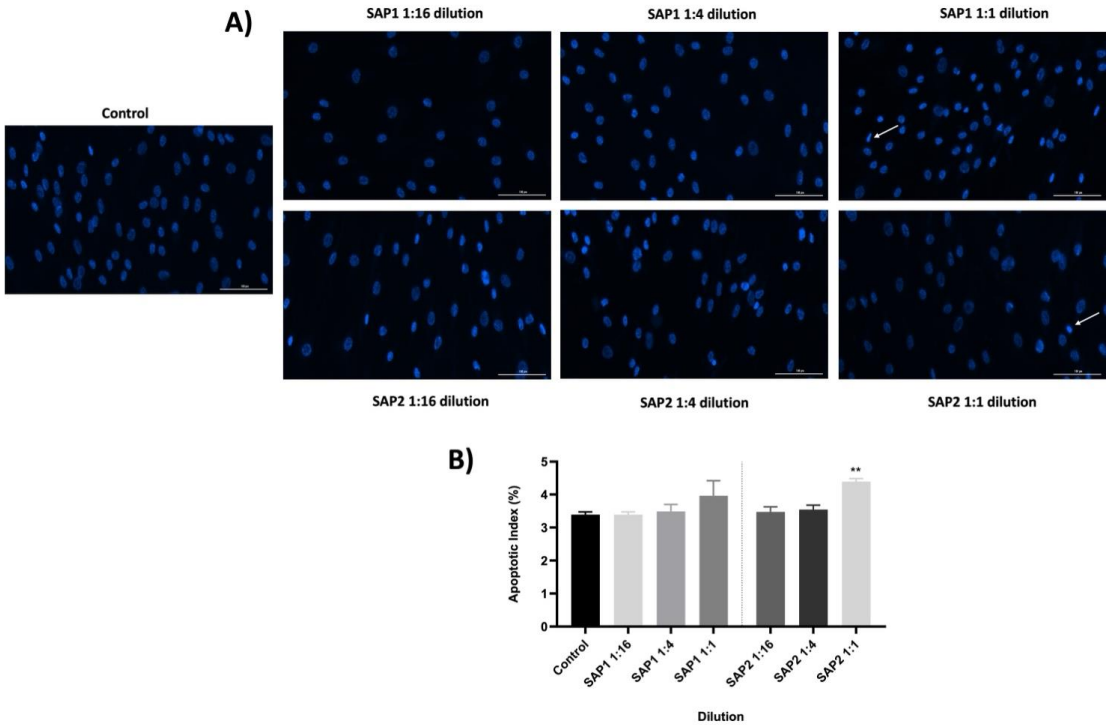

Figure 3.

(A) Representative pictures showing the changes in nuclear morphology at 72 h after treatment with SAP1 and SAP2 (1:16, 1:4, 1:1 dilution) in HGF cells. The images were captured at a magnification of 20×. The scale bar indicates 100 μm. (B) Graphical representation of the apoptotic index (%) in HGF cells 72 h after treatment with SAP1 and SAP2 (1:16, 1:4, 1:1 dilution)

All data are expressed as mean values ± SD obtained from three independent experiments performed in triplicate. For analysing the statistical differences between the control group and the treated groups, a one-way ANOVA test was conducted, followed by Dunnet's multiple comparison post-test; "*" indicates statistical significance (** $p < 0.01$)

The majority of previous studies examined the ability of self-assembling peptide-based dental repair agents to remineralise lesions, highlighting their ability to mimic extracellular matrix structures [24]. However, as far as we know, there are limited studies examining their impact at the cellular level and, in particular, at the level of the nuclei.

An advantage of combining *in vitro* and *in ovo* methods for determining toxicity is that additional information can be obtained regarding the safety profile of compounds that cannot be derived from *in vitro* methods alone. For example, information regarding the inflammatory response can be obtained using the hen's egg chorioallantoic membrane (CAM) method. Therefore, combining *in vitro* and *in ovo* methods provides a more comprehensive preclinical evaluation [32].

The next stage of the study involved evaluating the two gels' irritant and anti-irritant potentials at the level of the chorioallantoic membrane of the hen's egg.

SAP1 and SAP2 were tested in parallel with water, representing the negative control and SDS 1%, representing the positive control. An irritation score was calculated based on the appearance of vascular effects (haemorrhage, lysis and vascular coagulation) to quantify the irritating effect. Water did not cause any changes to the vascular plexus, whereas SDS 1% caused massive haemorrhages, coagulation and vascular lysis (Figure 4). In addition, the lowest irritation score was obtained for water (0.07), while the highest was obtained for SDS 1% (17.20). Both samples tested did not reveal significant capillary changes, but only mild intravascular coagulation and vascular lysis were observed. According to Table I, SAP1 has an irritation score of 1.57 and SAP2 has a score of 3.66.

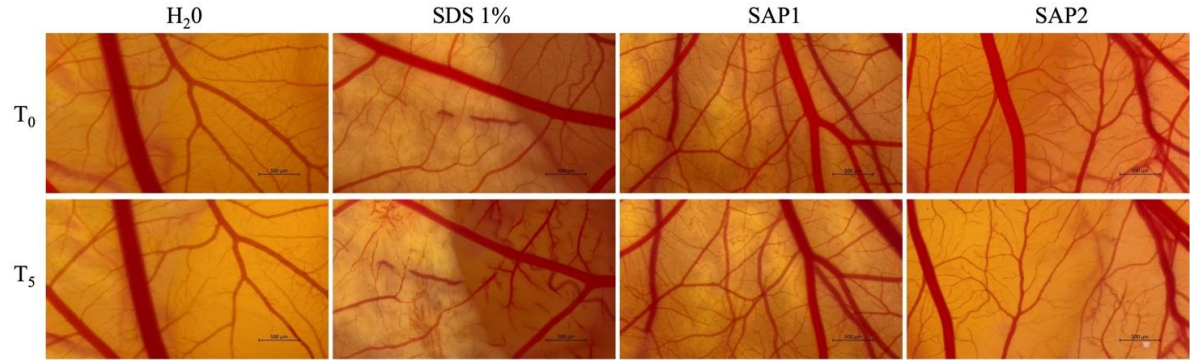

Figure 4.
Analysis of the irritant potential of SAP1 and SAP2 by the HET-CAM method
Stereomicroscopic images of CAMs inoculated with negative control – H_2O,
positive control – SDS and test compounds – SAP1 and SAP2

Table I
Irritation score values for positive control (SDS 1%), negative control (distilled water) SAP1 and SAP2

	H_2O	SDS 1%	SAP1	SAP2
IS	0.07	17.20	1.57	3.66
tH	300	60	300	300
tL	300	20	287	236
tC	300	80	260	230

Table II
Anti-irritant effect of SAP1 and SAP2

	SDS 1%	SAP1 + SDS 1%	SAP2 + SDS 1%
IS	18.01	5.40	6.22
tH	44	100	170
tL	70	240	160
tC	23	280	276
H_{AI}		2.27	3.86
L_{AI}		3.43	2.29
C_{AI}		12.17	12

In terms of their anti-irritant potential, both samples reduced the irritation score produced by SDS by 1% (5.40 for SAP1 and 6.22 for SAP2) (Table II) and the effects observed at the level of the vascular plexus.

For the positive control, SDS 1%, massive haemorrhages, coagulation and vascular lysis were observed. As a result of pre-treatment with SAP1 and SAP2, changes at the capillary level were significantly reduced (Figure 5).
HET-CAM is an alternative method for determining the irritant potential of different compounds without using live animals. The methodology is based on quantifying the irritating effect by calculating the irritation score. Substances are classified as non-irritating if IS value is between 0 and 0.9, irritating if it is between 1 and 8.9 and strongly irritating if it is between 9 and 21 [6]. According to this classification, we can conclude that the two samples are slightly irritating, but their irritation score indicates a good safety profile. Moreover, pre-treatment with SAP1 and SAP2 decreased the SDS's irritation score from 18.01 to 5.40 and 6.22, respectively.
HET-CAM is an appropriate method to determine the potential irritant properties of dental products owing to their direct contact with sensitive oral tissues. For example, cements containing calcium and silicate have been evaluated using this method, indicating that they can cause different degrees of irritation at the vascular plexus level [23].

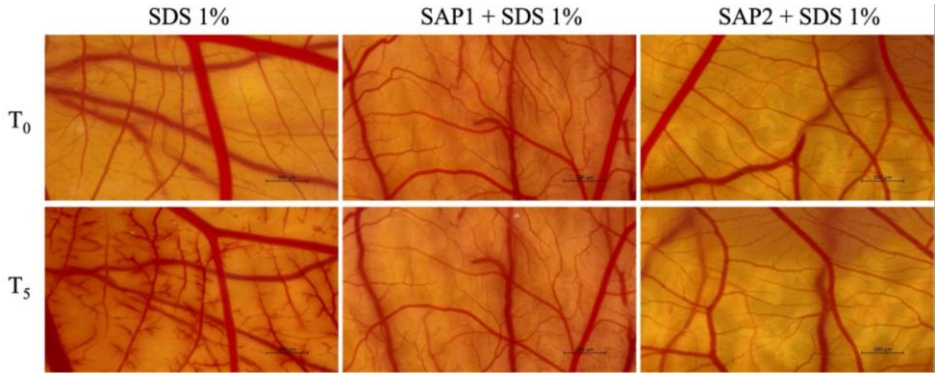

Figure 5.
Stereomicroscopic images of CAM after pre-treatment with SAP1 and SAP2 followed by 1% SDS irritation

In addition, chlorhexidine, widely used in dental practice as an antimicrobial agent, was evaluated for its irritant potential using chorioallantoic membranes. According to the study, chlorhexidine is irritating and severely

irritating depending on the concentration tested [15]. Similarly, sodium fluoride, xylitol and their combination, commonly found in dental products, have been tested *in ovo* for their irritant potential. It has been concluded from the study that none of the components have the potential to irritate [5]. To our knowledge, no *in ovo* studies have been conducted on the irritant potential of self-assembling peptide-based repair agents; this represents one novel aspect of the present study.

The differences in irritation scores and apoptotic indices between SAP1 and SAP2, with SAP2 being slightly more irritating, can be attributed to variations in their formulations. Although both gels share many common ingredients, SAP1 contains additional components such as sodium chloride and sodium sulphate that may stabilise the gel and influence its osmolarity, potentially reducing irritation by maintaining a more physiologically compatible environment. In contrast, the absence of these ingredients in SAP2 could make it less balanced regarding osmotic or buffering properties, potentially leading to higher irritation scores. These subtle differences in composition highlight how minor changes in formulation can impact the biological response. Further investigation into these ingredients' specific roles is necessary to understand their effects on irritation and cell apoptosis fully.

SAPs are designed to mimic natural processes in the body, mainly by forming nanostructures that promote tissue remineralisation and repair. However, the specific pathways through which they enhance cellular viability or minimise irritation are not detailed. One possibility is that SAPs create a protective scaffold, shielding cells from harmful environmental factors while providing structural support. Additionally, their interactions with cellular receptors or signalling pathways, such as those regulating oxidative stress or inflammation, could play a role in maintaining cell integrity. Their ability to stabilise ion concentrations and pH levels may also reduce irritation and enhance biocompatibility. Further studies are needed to elucidate these mechanisms, including how SAPs influence key cellular processes like apoptosis, proliferation and stress response.

Conclusions

The present study assessed the cytotoxicity of two gels composed of self-assembling peptides *in vitro* at the level of human gingival fibroblasts and *in ovo* at the level of the chorioallantoic membrane of chicken eggs. According to the study's results, both gels do not significantly affect cell viability, but the percentage of viable cells is not significantly affected. In addition, sampling did not alter the morphology of the cells, and there were no significant changes in the nuclei's structure. In terms of vascular irritation, neither gel induced signs of irritation at the level of the chorio-allantoic membrane, and the irritation scores obtained suggested that a minor irritation had occurred. Further, the two gels exhibited a protective effect at the level of the vascular plexus against the irritation produced by SDS when pre-treated with them.

In conclusion, both samples are ideal candidates for treating dentin demineralisation, given their excellent safety profiles *in vitro* and *in vivo*.

Conflict of interest

The authors declare no conflict of interest.

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