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Original Article

Influence of apigenin and seashell nanoparticles on the biological attitude of soft denture liner

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ABSTRACT

Objective: To determine the "biocompatibility" of a soft denture liner impregnated with apigenin and seashell nanoparticles via histopathological assessment in rabbit models.
Materials and Methods: Twenty-six albino rabbits aged 5–7 months and weighing, 1.25–1.5 kg were randomly divided into apigenin and seashell-modified groups, n = 13 each). A total of 104 disc-shaped specimens (3 mm thick and 5 mm in diameter) of a heat-cured soft liner, (GC, USA) were prepared. In the apigenin group, each rabbit was subcutaneously implanted with 4 specimens: (positive control, unmodified-liner, and 0.25 %, 0.5 %, and 1 % of apigenin-modified liner). In the seashell group, rabbits were implanted with positive control and 1.25 %, 2.5 % and 5 % seashell-modified liner. A non-implanted incision was performed for all the groups as a negative control. Histopathological observations were evaluated according to inflammatory and angiogenesis scores 14 days after implantation.
Results: A significant decrease in inflammatory responses and an increase in angiogenesis were observed for both apigenin and seashell-modified soft liner groups. Higher compatibility effectiveness was positively related to and recorded in the increased ratios of nanoparticles within the soft liner material.
Conclusion: Apigenin and seashell-modified soft liners at higher ratios 1% and 5% respectively were more compatible with the rabbit mucosa.

1. Introduction

Soft denture liners make denture wearing more comfortable because of their rehabilitating effect of uniformly distributing masticatory forces on the basal seat mucosa. As such, these liners have been used to treat traumatized denture-bearing mucosa, severe bony undercuts, edentulous arches opposing natural dentition, xerostomia, oral defects requiring obturations, and advanced alveolar ridge resorption where patients cannot tolerate hard denture bases (Borg, 2021). However, these liners are associated with several limitations during clinical use that affect their serviceability, including the sorption of oral fluids and colonization by pathogenic microorganisms (Elawady et al., 2019).

Many attempts have been made to overcome these drawbacks, and the most effective approach is the incorporation of nano-particles to obtain the benefits of their superior properties (Akay and Avukat, 2019). Chemical nano-particles, such as Zirconium, Titanium, and Silver have been widely used with soft liners to reduce the microbial load and improve liner properties (Ahmed and Ali, 2018).

Incorporation of natural pharmaceutical nano-particles into soft denture liners is crucial. Apigenin, a naturally occurring trihydroxy flavone, found mostly in vegetables and fruits, such as tea, parsley, oranges, and onions. (Wang et al., 2019), has drawn interest for its numerous bioactivities, including antibacterial, antiviral, antifungal, antiparasitic, in addition to anti-oxidant, anti-mutagenic, anticarcinogenic, and anti-inflammatory effects (Morimoto et al., 2015). Medical researches have shown that apigenin reduced glucose levels, accelerated wound healing, and reduced free radicals via oxidative stress. (Ibrahim et al., 2020a; Ibrahim et al., 2020b; Rajab et al., 2022).

A seashell, is a mollusk's protective shield, such as a clam or oyster (Moss et al., 2019) predominantly composed of calcium and phosphate with a natural ceramic structure similar to hard biological tissue constituents such as teeth and bones; as such, they are excellent sources of

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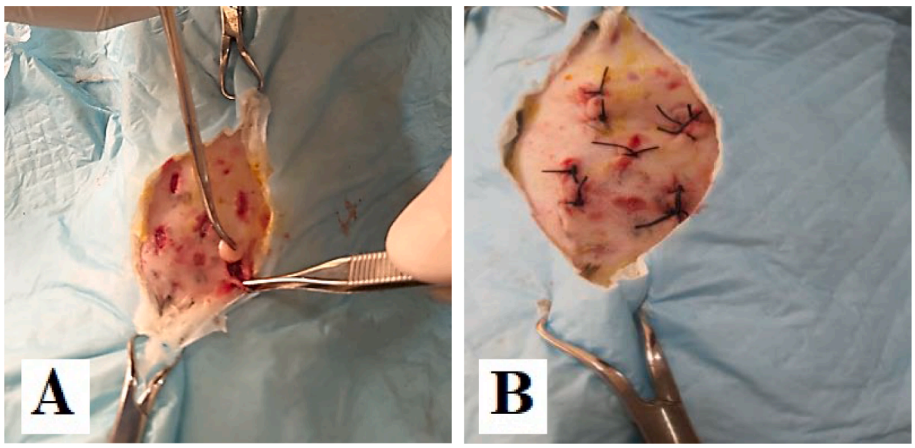

Fig. 1. Surgical steps of the study. A: specimens implantation. B: suturing.

numerous compounds including, hydroxyapatite (Orman et al., 2019), and many oxides mainly those of calcium, magnesium, and trace amounts of iron oxide. Seashells have thus been used in the preparation of hydroxyapatite and calcium-based dental cements; and have demonstrated antimicrobial efficacy against oral *Lactobacilli*, *Streptococcus mutans*, and *Enterococcus faecalis* (Al-Yousifany et al., 2015; Alhussary et al., 2020).

The incorporation of nano-sized natural materials into soft liners requires to investigate their biocompatibility in vivo including cytotoxicity analysis and potential toxicity assays, such tests may be conducted on subcutaneous tissues by investigating the reaction of animal cells to the implanted material (Al-Ali and Kassab-Bashi, 2015; Tsuji et al., 2015).

This study aimed to histologically determine the biocompatibility of a soft liner modified with seashell and apigenin nanoparticles in rabbit experimental models. The null hypothesis was that the modification of the soft liner through different ratios of nanoparticles would not lead to enhancing the liner compatibility.

2. Materials and methods

2.1. Soft denture liner preparation

A total of one hundred and four disc-shaped specimens (5 mm diameter and 3 mm thick) of super-soft acrylic heat-activated soft liner, GC, USA were prepared by mixing of 5 gm of powder with 4 ml liquid in compliance with the manufacturer's guidelines. Apigenin nano powder at (0.25, 0.5 and 1 %), and seashell nano powder at (1.25, 2.5 and 5 %) ratios were added to the liner liquid, which represented the minimum inhibitory concentrations, (MIC) against *Candida albicans* (Jaffer et al., 2023). The liner's powder weight was deducted from the additive weight and mixed with the liner's liquid within a sonicator to disperse the granules evenly, the liquid and soft liner's powder were mixed, and covered until they formed dough which was then packed into previously prepared stone molds with the required specimens dimensions. Curing was carried out in hot water at 165°F for 30 min followed by additional 10 min of boiling, according to the manufacturer' guidelines.

2.2. Experimental animals

Twenty-six healthy albino male rabbits aged 5–7 months and weighing 1.25–1.5 kg were divided into two groups according to the kind of additive, (apigenin and seashell). Four specimens were subcutaneously implanted in each rabbit; three of them represented the three ratios of nano-additive in addition to a specimen (positive control) without any additives. The animals were housed in well ventilated animal house. They were fed with a normal diet and water thrice a day throughout the study period.

2.3. Anesthesia

Intramuscular injection of a mixture of 1.3 ml ketamine hydrochloride, general anesthesia (40 mg/kg) and 0.3 ml xylazine, sedative, and analgesic (5 mg/kg) was applied to anaesthetize animals within approximately 5 min and maintained for about 40 min (Ahirwar et al., 2021).

2.4. Specimens' implantation and post-operative care

The fur in the specific surgical site (distal dorsum) was shaved to create an area for implantation in which the upper right side represented the positive control specimen, whereas the upper left and the two lower sites represented ratios of the modified soft liner. An incision was made in the middle of the surgical site without implantation (negative control). The surgical area was disinfected with 5 % povidone-iodine solution. A detachable blade no.15 on a scalpel was used to make a 10 mm incision in the skin, with a 10 cm distance between incisions. A pocket was created subcutaneously utilizing blunt dissection for the implanted specimen, which was placed in the pockets 10 mm away from the incision line using tweezers, and the skin was sutured with a 3.0 black silk suture Fig. 1. The procedure was performed by a veterinarian. Following surgery, the rabbits were maintained in isolated cages, and a normal diet protocol was followed throughout the experimental period.

2.5. Histopathological assessment

After 14 days of the experimental period, biopsy specimens from the implantation site and surrounding subcutaneous tissue were taken for histopathological analysis. The sectioned tissue was fixed in 10 % formalin, treated in alcohol for 48 h, embedded in paraffin wax blocks and then sectioned by microtome into 5 μm thick sections. Staining was performed using haematoxylin and eosin. Slide examination was performed microscopically, and histopathological observations were accomplished according to the special criteria of inflammation and angiogenesis response scoring.

2.5.1. Inflammatory cells infiltration scoring

After staining, the number of inflammatory cells in the surgical field was counted according to a previously published protocol (Gupta and Kumar, 2015):

Score 1: "Nil: no inflammatory cells are present."

Score 2: "Mild: few cells are present, less than ½ of the operation field."

Score 3: "Moderate: moderate number of cells is present in more than

Table 1
Kruskal- Wallis test of inflammation and angiogenesis in apigenin-modified liners.

Groups Criteria	Negative control group incision without implanted material	Positive control group without nanoparticles	0.25 % Apigenin group	0.5 % Apigenin group	1 % Apigenin group	P-Value
Inflammation	2.33 ± 0.33 ABC	3.00 ± 0.00 AB	2.66 ± 0.33 ABC	2.00 ± 0.33 C	0.66 ± 0.33 D	<0.001
Angiogenesis	1.66 ± 0.33 A	1.66 ± 0.33 A	2.66 ± 0.33 A	3.00 ± 0.00 B	1.66 ± 0.33 A	0.026

Data are expressed as mean ± standard deviation.
Significant differences are indicated by different letters in rows at $p \leq 0.05$.

Table 2
Kruskal- Wallis test of inflammation and angiogenesis in seashell-modified liners.

Groups Criteria	Negative control group incision without implanted material	Positive control group without nanoparticles	1.25 % seashell group	2.5 % seashell group	5 % seashell group	P-Value
Inflammation	0.66 ± 0.33 A	2.66 ± 0.00 B	2.66 ± 0.33 B	1.33 ± 0.33 A	1.00 ± 0.00 A	0.006
Angiogenesis	0.33 ± 0.33 A	1.33 ± 0.33 B	1.66 ± 0.33 B	1.66 ± 0.00 B	2.66 ± 0.33 C	0.003

Data are expressed as mean ± standard deviation.
Significant differences are indicated by different letters in rows at $p \leq 0.05$.

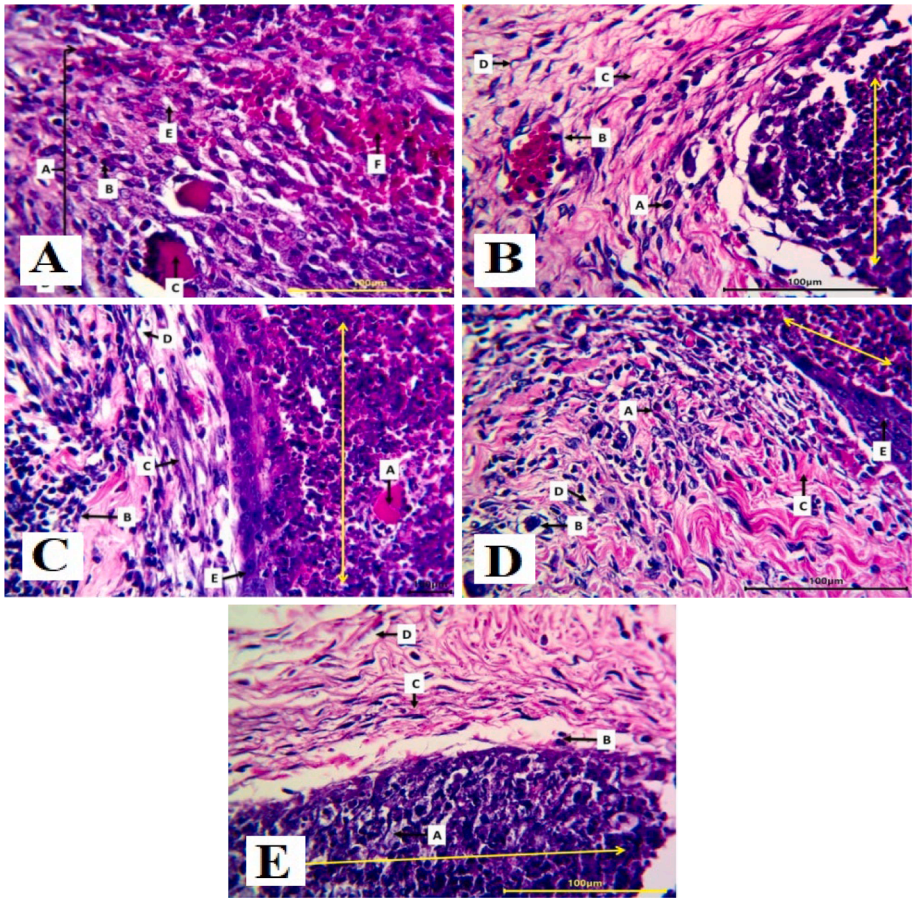

Fig. 2. Histopathological behaviors of apigenin-modified soft liner (400X). **A**: negative control, with inflammatory cells. **B**: positive control, high inflammation, and angiogenesis. **C**: 0.25% apigenin-modified liner, high inflammation, and angiogenesis. **D**: 0.5% apigenin-modified soft liner, moderate inflammation, and good angiogenesis. **E**: 1% apigenin-modified soft liner, mild inflammation, and angiogenesis.

½ of the operation field."

Score 4: "Abundant: many cells are present in more than ¾ of the operation field,"

2.5.2. Angiogenesis scoring

Angiogenesis was scored according to (Rahman et al., 2019), as follows:

Score 0: "None: absence of angiogenesis, including congestion and

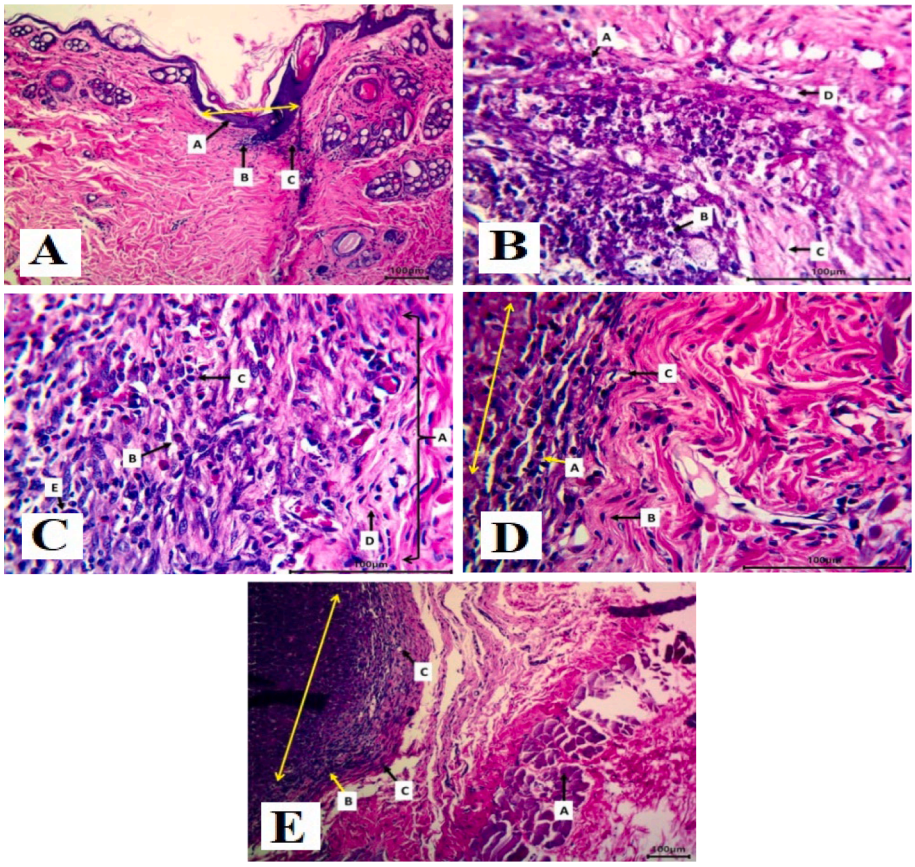

Fig. 3. Histopathological behaviors of seashell-modified soft liner (400X). **A**: negative control, slight inflammation. **B:** positive control, high inflammation, and angiogenesis. **C:** 1.25% seashell-modified soft liner, high inflammation, and angiogenesis. **D:** 2.5% seashell-modified soft liner, moderate inflammation, and good angiogenesis. **E:** 5% seashell-modified liner, mild inflammation and angiogenesis.

hemorrhage."

Score 1: "Minimum: 2–4 vessels per site, congestion and hemorrhage."

Score 2: "Mild: 4–6 vessels per site, slight congestion."

Score 3: "Sever: 7–8 vessels per site vertically disposed toward epithelial surface."

2.6. Statistical analyses

Descriptive statistics were conducted using SPSS software (Version 25); the variance between the study groups was analyzed by Kruskal-Wallis test with a significance threshold of $P \leq 0.05$.

3. Results

Significant differences in the inflammatory response and angiogenesis among the groups of apigenin-modified liners are shown in Table 1. Minimum inflammation was observed for the 1 % apigenin group and the highest for the positive control group. Maximum angiogenesis was recorded for the 0.5 % apigenin group. Moreover, a significant difference was observed among the seashell-modified liner groups (Table 2). The lowest level of inflammation was detected for the negative control followed by the 5 % seashell group which also showed the highest level of angiogenesis.

Histological findings were reported using photomicrographs as shown in Fig. 2 and Fig. 3. Each figure shows five observations: A: negative control, non-implanted incision, B: positive control, unmodified liner, and C, D, and E in Fig. 2 show apigenin addition at 0.25 %, 0.5 %, and 1 %, respectively, and in Fig. 3, seashell addition at 1.25 %, 2.5 %, and 5 %, respectively. The inflammatory responses were monitored by the infiltration of polymorpho-nuclear, mononuclear inflammatory cells and giant cells surrounded by fibrous tissue. Conversely, angiogenesis was associated with hemorrhage in sever responses accompanied by full re-epithelialization in the highest ratio of the additive material.

4. Discussion

Experimental animal tests are generally considered to be acceptable for assessing dental material compatibility prior to use in humans. In the present study, experimental rabbits were selected because of their histological similarity in skin structure and buccal mucosa to humans (Li et al., 2016).

Overall, the results of this study showed that, the highest ratios of apigenin and seashell nano-additives illustrated greater compatibility in the inflammatory-index; therefore, the null hypothesis was rejected.

Subcutaneous implantation of the apigenin-modified soft liner revealed significantly fewer inflammatory responses and higher angiogenesis than non-modified materials, indicating the pharmaceutical effectiveness of apigenin. Apigenin is a cyclooxygenase blocker flavonoid that exhibits potent activity against edema and other inflammatory reactions (Al-Khayri et al., 2022). In this study, the results were expressed as a function of apigenin via binding and inhibition of the activity of the oncogenic kinase Src (a non-receptor tyrosine) responsible for the inflammatory reaction. The inhibition of Src plays a significant role in inflammation and cancer inhibition because it results in suppression of the signaling pathways to reduce COX-2 expression, which is involved in carcinogenesis and skin inflammation (Wang et al., 2019).

Additionally, apigenin improves epidermal permeability barrier

function, which inhibits acute inflammation and inhibits changes in skin surface pH by decreasing transepidermal water loss. Moreover, apigenin inhibits inflammation by reducing levels of matrix metalloproteinase-1, and those of TNF-alpha and IL-6, the proinflammatory cytokines, (Man et al., 2012), besides an effective role in triggering the Nuclear Factor Erythroid 2-Related (NrF-2), which controls the cloning of genes responsible for manufacturing antioxidant producing enzymes such as glutathione (Ibrahim et al., 2020b).

Apigenin has also been shown to facilitate wound healing by enhancing the re-epithelialization and deposition of collagen fibers in the dermis, in addition to its efficiency in granulation tissue formation. Furthermore, it has been shown to exert an inhibitory effect on lipid peroxidation, which resulted in improvements of the viability of collagen fibril by promoting DNA synthesis, reducing cell damage, and enhancing wound healing by increasing blood vessels and collagen fibers (Rajab et al., 2022; Shukla et al., 2016).

The positive histological findings of the seashell-modified soft liner found in this study are related to the action of the seashell as a bio-efficient material and a biomineralizer owing to its high mineral content, and has been applied for anticancer and antibiotic drug delivery with an effective role in osteoporosis therapy and bone remodeling and as an alternative source of synthetic grafting materials (Mailafiya et al., 2019; Chakraborty et al., 2020; Desmond et al., 2020). Furthermore, hydroxyapatite from seashells has been shown to exert a strong antimicrobial and antibiofilm effect against *E. coli, S. aureus, B. subtilis, K. pneumonia,* and *C. albicans*, rendering the material a solution to overcome multidrug- resistant bacteria (Ahmed et al., 2022).

Seashell hydroxyapatite also exhibited a high anti-inflammatory effect and restricted infiltration of inflammatory cells in the mandibular bony defect owing to its role in releasing anti-inflammatory cytokines stimulators (Shapovalova et al., 2016), and supporting bone growth and healing (Orman et al., 2019; Alhussary et al., 2020).

The limitations of this study are that: only one type of soft liners was tested, and that properties other than compatibility were not fully assessed. Therefore, further studies of the material physical properties are recommended.

5. Conclusion

The addition of apigenin and seashell nanoparticles to acrylic-based soft liner was found to show a positive biological activity in the experimental animals, and histological analysis showed anti-inflammatory effects and enhancement of angiogenesis at the highest ratios of the nanoparticles.

CRediT authorship contribution statement

Nadia Tawfiq Jaffer: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Rizgar Mohamed-Ameen Hasan:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **GhadaAbd Alrhman Taqa:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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.

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Ethical approval

This study was approved by the institutional protocols of the Ethics Committee Board of the College of Dentistry-University of Duhok, and the Ministry of Health, Kurdistan Region-Iraq (approval number 1011 on 23-11-2021).

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