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Original Article

Dentistry Section

Comparative Evaluation of Anti-bacterial, Anti-inflammatory Efficacy and Cytotoxicity of Triple Antibiotic Paste Modified Soft Liners with Conventional Soft Liners: An In-vitro Study

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ABSTRACT

Introduction: Soft-Liner is an acrylic temporary relining material for the temporary rebasing (relining) of acrylic dentures and tissue conditioning. The use of soft liner as a medium to deliver anti-bacterial and anti-inflammatory medications locally to the site enhances the healing of the soft tissues, further maintaining the mucosa healthy in the transitional healing period. Soft liners modified with Triple Antibiotic Paste (TAP) help in soft tissue healing and reduce post-surgical inflammation.

Aim: To evaluate the anti-bacterial, anti-inflammatory efficacy, and cytotoxicity of TAP modified soft liners with conventional soft liners.

Materials and Methods: The in-vitro study was conducted in the Gold Laboratory at Saveetha Dental College, Chennai, Tamil Nadu, India, in March 2023. A solution of TAP was prepared and mixed with Gas Chromatography (GC) soft liner material. The antibacterial efficacy against the strains of *Staphylococcus aureus, Pseudomonas*, and *E. faecalis* was assessed, and Mueller Hinton Agar was used to identify the zone of inhibition. The Kolmogorov-Smirnov test was conducted to assess the normality of the distribution, and non-parametric tests were performed for further analysis. A chi-square test was used to compare the cytotoxicity of TAP modified soft liners, which was assessed by a Lethality assay for brine shrimps over 24 hours. The Egg albumin denaturation assay was used to assess anti-inflammatory properties, with different concentrations of 10 µL, 20 µL, 30 µL, 40 µL, and 50 µL. Human Gingival Fibroblast (HGF) was used for the cell line study, and the isolation of HGF was performed by enzymatic digestion subjected to collagenase (900 u/mL) and dispase (400 u/mL) digestion at 37°C for one hour.

Results: In comparing the modified TAP liners to commercially available liners on the basis of antibacterial efficacy, there was increased anti-bacterial efficacy in the TAP modified liners, which increased with increasing concentration, with the maximum being 40.25±14.87 mm for a 1:3 concentration against *S. aureus* and the least being 23±1.3 mm of unmodified soft liners against *Pseudomonas* and *S. aureus*. Different concentrations of 10 µL, 20 µL, 30 µL, 40 µL were used for the anti-inflammatory test, and as the concentration increased, anti-inflammatory activity also increased. The cytotoxicity of the material increased from 10% to 40% as the concentration of TAP rose from 5 µL to 80 µL.

Conclusion: TAP shows a better response in managing postoperative inflammation and better soft tissue healing when incorporated into the soft liners. More precise studies are needed to understand the exact mechanism of TAP.

Keywords: Antibacterial activity, Dental materials, Nano-technology in dentistry

INTRODUCTION

Soft liners are materials used in dentistry to improve the fit and comfort of dentures. These soft liners conform to the shape of the gums and provide cushioning between the denture and the underlying tissues. They can be used for a variety of reasons, such as to provide relief from sore spots caused by denture sores, to improve the fit of an existing denture that has lost stability over time, or to provide a temporary cushion for new dentures while the gums heal and adjust [1,2].

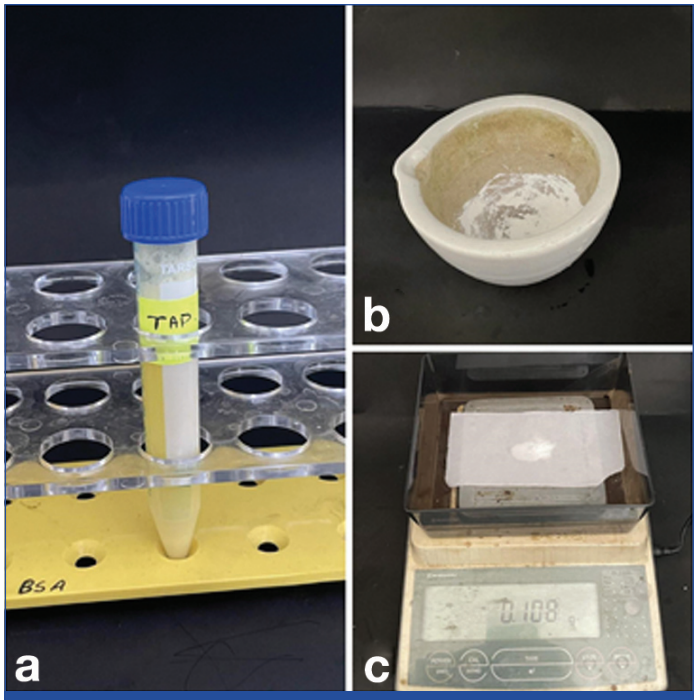
There are two types of soft liners: temporary and permanent. Temporary soft liners are typically used for a short period of time while the gums heal and adjust to a new denture; they are also known as tissue conditioners. Permanent soft liners, on the other hand, are designed to last for a longer period of time and can be used to improve the fit and comfort of an existing denture. Soft liners can also be used after implant surgery to help protect the implant site and improve patient comfort during the healing process. In this case, a temporary soft liner is typically placed over the implant site to provide cushioning and protection while the implant fuses with the jawbone [2,3].

The soft liner can also help to distribute pressure more evenly across the implant site, reducing the risk of implant failure or complications. Once the healing process is complete, the soft liner can be removed and replaced with a more permanent restoration such as a crown or bridge. Improper use or maintenance of soft liners can lead to bacterial growth and infection, which can compromise the healing process and lead to further complications.

Soft liners are typically made from a variety of materials, including silicone, acrylic, and thermoplastic elastomers. Each material has its own unique properties and benefits. Silicone is a popular soft liner material due to its biocompatibility and softness. It is also resistant to bacterial growth and easy to clean. Silicone liners are often used for long-term wear as they are durable and can maintain their shape over time. Acrylic soft liners are often used as a temporary solution as they are easier to apply and can be easily adjusted as needed. They are also relatively inexpensive compared to other soft liner materials. Thermoplastic elastomers are a newer type of soft liner material that can be molded to fit the contours of the patient's gums. They are often used in cases where a precise fit is required or where the patient has thin, delicate mucosa [1,4,5].

As soft liners offer several advantages as an adjuvant before the delivery of permanent prosthesis, the necessity for them to possess antimicrobial properties becomes apparent. TAP is a mixture of three different antibiotics commonly used in endodontic (root canal) treatment to eradicate bacterial infections in the root canal system. The three antibiotics typically used in TAP are ciprofloxacin, metronidazole, and minocycline. Ciprofloxacin is an antibiotic commonly used to treat urinary tract infections, respiratory infections, and skin infections caused by bacteria. Metronidazole is an antibiotic effective against anaerobic bacteria, which are frequently found in dental infections. Minocycline is a broad-spectrum antibiotic commonly used to treat acne and other bacterial infections [5,6].

Previous studies were conducted by modifying soft liners with various anti-fungal agents for lining onto the dentures to eliminate denture sores and target activity against *Candida albicans*. Another study by Baygar T et al., utilised carvacrol with soft liners to improve antimicrobial activity and concluded that the incorporation of carvacrol decreased biofilm formation by 98.03±0.2%. The inhibition zones were measured at 43.67±0.58 mm and 40.33±0.58 mm against *Bacillus subtilis* and *Streptococcus mutans* [7,8]. This study aimed to address the anti-bacterial properties of soft liners, incorporating modified soft liners for post-surgical implant soft-tissue healing and to assess the anti-bacterial efficacy, anti-inflammatory efficacy and cytotoxicity of the modified soft liner.



[Table/Fig-1]: a) TAP mixed with 10 mL distilled water; b) Triple Antibiotic Paste (TAP), and c) Soft liner polymer.

MATERIALS AND METHODS

The in-vitro study was conducted in the Gold Laboratory (Nanobiomedicine Lab) at Saveetha Dental College, Chennai, Tamil Nadu, India, in March 2023. This study was cleared by the ethics committee of Saveetha Dental College. Sample size estimation and calculation were performed using the study by Bertolini MM et al., as the reference article with G*power software. The study protocol was approved by the Institutional Ethics Committee with the reference number IMPLANT/2209/23/TH-020. The preparation of the modified "TAP liners" and the sequential tests that were conducted are detailed below [9].

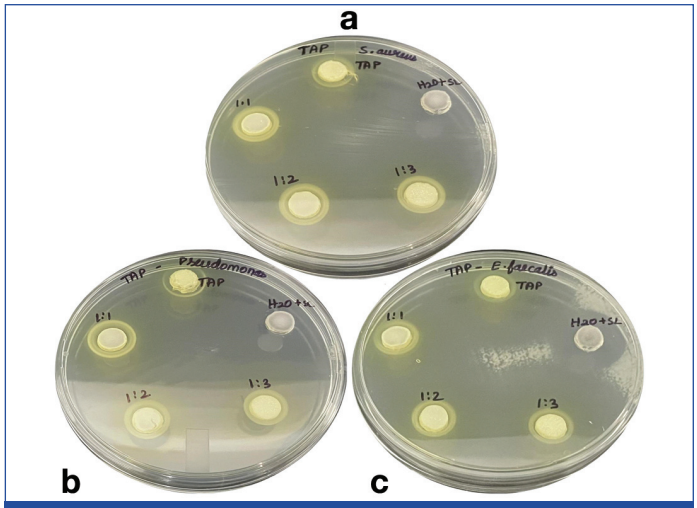
Preparation of TAP (Triple Antibiotic Paste)

The paste for this study was made using 50 mg of minocycline, 500 mg of ciprofloxacin, and 400 mg of metronidazole in a definite proportion of 1:1:1. The three drugs were ground using a mortar and pestle to create a uniform powder, which was then dissolved in 10 mL of distilled water [Table/Fig-1a,b]. The solution was allowed to settle for a few hours, resulting in the final mixture used as a triple antibiotic solution in this study. GC soft liner material manufactured by GC Dental Products Corporation India was then mixed in proportions of 1:1, 1:2, and 1:3 (1:1-0.1 g of soft liner, 1 mL of TAP solution; 1:2-0.1 g of soft liner, 2 mL of TAP solution; 1:3-0.1 g of soft liner, 3 mL of TAP solution) [Table/Fig-1c] [10].

Antibacterial Activity

The varying concentration of the modified liners' antibacterial efficacy against the strains of *Staphylococcus aureus, Pseudomonas*, and *E. faecalis* was assessed. These organisms were particularly chosen as they are crucial for the propagation of infection immediately after stage 1 implant surgery. The newly formulated TAP liners were developed for use around dental implants during healing periods [11-13].

For this, Mueller Hinton Agar was used to identify the zone of inhibition. Mueller Hinton agar was prepared for fifteen minutes at 121 degrees Celsius and sterilised. Sterilised plates were filled with media, which was then left to solidify. A 9 mm sterile polystyrene tip was used to cut the wells, and the test organisms were swabbed. The modified soft liners at various concentrations (1:1, 1:2, 1:3, TAP alone, and soft liner alone) were loaded in five wells. The plates were then incubated at 37°C for 24 hours. The zone of inhibition was assessed following the incubation period [Table/Fig-2] [14-17].

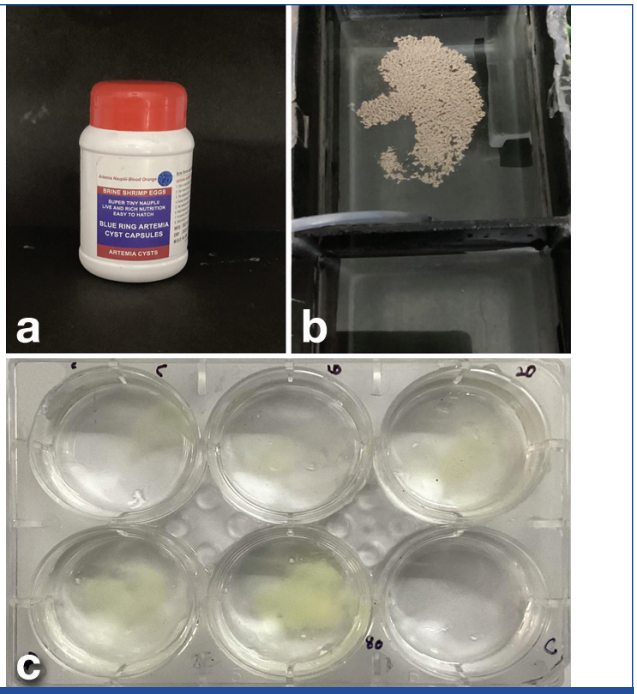


[Table/Fig-2]: a) TAP against *S. aureus*; b) TAP against *E. faecalis*, and c) TAP against *Pseudomonas*.

Cytotoxicity Test

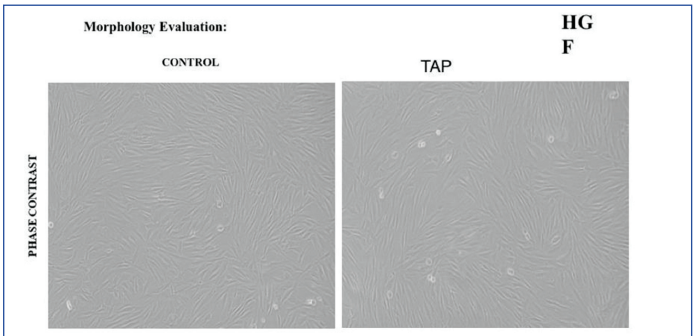
Brine shrimp lethality assay: A lethality assay for brine shrimps was conducted to assess the cytotoxicity of the modified soft liners [Table/Fig-3a]. A total of 200 mL of distilled water was used to dissolve 2g of iodine-free salt [Table/Fig-3b]. A total of 10-12 mL of saline water was added to six Enzyme Linked Immuno-sorbent Assay (ELISA) plates [Table/Fig-3c]. This was followed by the progressive addition of 10 nauplii (5 µL, 10 µL, 20 µL, 40 µL, 80 µL, and control) to each well. The soft liners containing antibiotics were then added in a 1:3 concentration of soft liners to antibiotics, as this concentration provided improved results in the antibiotic analysis. The plates were then incubated for 24 hours. After 24 hours, the ELISA plates were examined and counted, and the number of living nauplii present was estimated using the formula: 100 divided by the product of the number of dead nauplii and the number of live nauplii [18,19].

Cell line study: HGF Cells were isolated from normal adult humans (aged from 18 to 25 years) gingival tissue at Saveetha Dental College and hospitals. The isolation of HGF was performed by enzymatic digestion, and subjected to collagenase (900 u/mL) and dispase (400 u/mL) digestion at 37°C for one hour. Primary dental pulp cell cultures were carried out with Roswell Park Memorial Institute (RPMI) 1640 (In-vitrogen Corporation, CA, USA) supplemented with



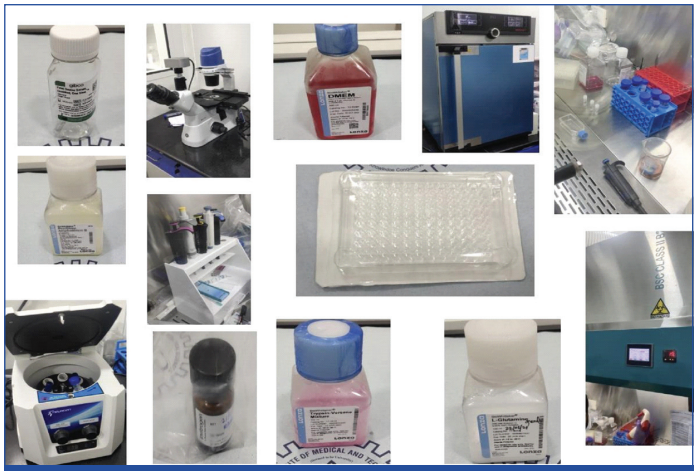
[Table/Fig-3]: a) Brine shrimp eggs; b) Brine shrimp eggs mixed with salt water, and c) Ten live nauplii in each TAP concentration.

20% (v/v) foetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5% CO_2 [Table/Fig-4]. The armamentarium needed for the cell line study is depicted in [Table/Fig-5]. The culture medium was changed every three days and sub-cultured at 80% confluence. At passage 2, cells were seeded in culture dishes for all in-vitro experiments in this study [20,21]. To demonstrate the repeatability of the results of the specific concentration of the TAP Soft liners, triplicate sampling was carried out. A 1:3 concentration of TAP Soft liners was assessed for cell viability at varying percentages like 2%, 5%, 10%, 15%, 20%, and 40%, and the samples were triplicated to assess repeatability and minimise errors.



[Table/Fig-4]: Fibroblastic cell line morphological analysis between control (untreated soft liners) and TAP (1:3) modified group.

Cell Viability/MTT assay: To assess the biocompatibility/cell viability of the antibiotic formulation TAP (TAP-minocycline 50 mg, ciprofloxacin 500 mg, metronidazole 400 mg) on HGF cells for 24 hours, cell viability was evaluated by MTT assay as previously described. Briefly, after exposure to elutes of different percentages (2%, 5%, 10%, 15%, 20%, and 40%) of untreated soft liners in the control group and the experimental group TAP (1:3) of antibiotics, HGF cells were seeded in a 96-well culture plate for 24 hours. To determine percent viability, the post-incubated cells were treated with 10 µL of stock MTT dye (10 mg/mL) in each well, and the plate was incubated again at 37°C for four hours. The medium was then replaced with 100 µL of DMSO (Dimethyl sulfoxide) in each well to dissolve the formazan crystals, and absorbance was recorded at 570 nm using the Synergy Hybrid Multi-Mode Reader (BioTek, Winooski, VT, US). The percent cell viability was calculated using the following equation:

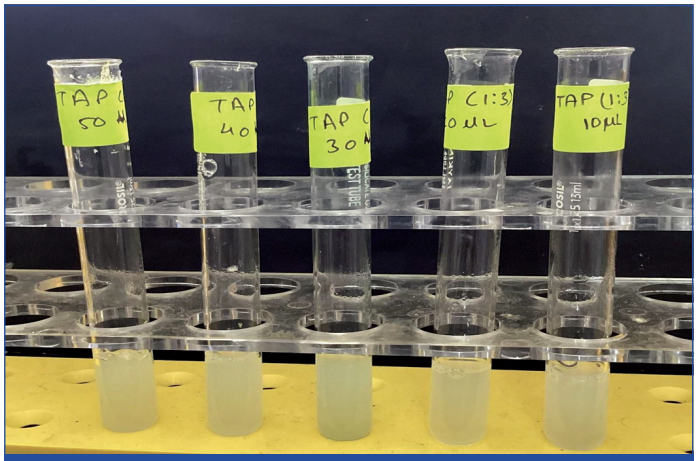


[Table/Fig-5]: Armamentarium needed for cell line study.

Cell Viability (%)=O.D. of cells treated with CLC NP s/O.D. of control cells×100 [20].

Anti-inflammatory Test

Egg albumin denaturation assay: A 5 ml solution was prepared, containing 2.8 mL of freshly manufactured phosphate-buffered saline with a pH of 6.3 and 0.2 ml of egg albumin from hens' eggs. For soft liners containing antibiotics (1:3 concentration), the concentrations taken individually were 10 µL, 20 µL, 30 µL, 40 µL, and 50 µL. A positive control, diclofenac sodium, was employed. The mixes were then cooked in a water bath for 15 minutes at 37µ. The samples were allowed to cool to ambient temperature, and the absorbance at 660 nm was measured [Table/Fig-6] [22-24].



[Table/Fig-6]: TAP with different concentrations.

STATISTICAL ANALYSIS

The Kolmogorov-Smirnov test was conducted to assess the normality of the distribution. Non-parametric tests were then performed to analyse the variables. A Chi-square test was conducted to compare the cytotoxicity of TAP modified soft liners, which was assessed by a lethality assay for brine shrimps over 24 hours.

RESULTS

Antimicrobial Test

The antimicrobial test conducted in this study revealed that TAP had a zone of inhibition measuring 34.0±1.06 mm, 34.87±1.55 mm, and 32.87±1.12 mm against anaerobic bacteria, namely *E. faecalis, Pseudomonas*, and *S. aureus*, respectively. The unmodified soft liner showed a zone of inhibition measuring 23.75±2.65 mm, 23.00±1.30 mm, and 23.87±1.80 mm. The variable ratios of TAP-modified soft liners, 1:3, showed the maximum zone of inhibition of 40.25±14.87 mm against *S. aureus* and the least inhibition in the 1:1 concentration with *Pseudomonas*. The significance values and the mean±SD have been tabulated below in [Table/Fig-7].

	E. faecalis	*Pseudomonas*	*S. aureus*	Significance
Tap (plain)	34.0±1.06	34.87±1.55	32.87±1.12	0.74
Soft liners (plain)	23.75±2.65	23.00±1.30	23.87±1.80	0.59
Tap:soft liner (1:1)	34.37±1.40	30.75±0.88	31.87±1.24	**<0.001***
Tap:soft liner (1:2)	35.87±1.45	32.00±1.30	33.62±0.91	**<0.001***
Tap:soft liner (1:3)	37.37±1.06	33.62±1.06	40.25±14.87	**<0.001***

[Table/Fig-7]: Comparison of antimicrobial activity by zone of inhibition (in mm) against three different bacteria.
*p-value is significant; Test used: One-way Analysis of Variance (ANOVA)

Cytotoxicity Test

Brine shrimp lethality assay: The cytotoxicity test conducted using Nauplii eggs indicated that as the concentration of TAP increased, the cytotoxicity of the material also increased. The control group, using distilled water, showed no egg mortality on day one and day two. However, at concentrations of 5 µL and 10 µL, no eggs died on day 1 except for the 1:3 TAP:Soft liner concentration, which had a mortality rate of 98% on day 2. Further increasing the concentration to 20 µL, 40 µL, and 80% resulted in a further reduction in egg viability. Specific values are provided in [Table/Fig-8].

Testing group	Concentration for testing				
	5 µL	10 µL	20 µL	40µL	80 µL
Control	100	100	96.2	92.52	78.5
1:3 (Soft liner: TAP)	100	98	96	89	77

[Table/Fig-8]: Mean percentage of live nauplii at varying concentrations of TAP; soft liner usage for a cytotoxicity test demonstrated that higher concentrations reduce nauplii viability.

Cell line study: The viability of HGF fibroblast cells was assessed in the cell line study. At a concentration of 1:3 TAP:Soft liners, the cell viability decreased to 95%. Subsequently increasing the concentration of TAP:Softliners to 5% and 10% further decreased the viability by 2% with each concentration increase. A similar trend was observed with increasing concentrations to 15% and 20%, with viability decreasing by 2% at each concentration increment. Upon further increasing the concentration to 40%, cell viability dropped to 90%, demonstrating a consistent decrease in viable cells with increasing TAP concentration. Details are provided in [Table/Fig-9].

Varying Concentrations of TAP: Soft Liners: % of Cell Viability						
Concentration	2%	5%	10%	15%	20%	40%
Reading 1	98	96	94	92	90	88
Reading 2	98	96	94	92	90	88
Reading 3	98	96	94	92	90	88

[Table/Fig-9]: Percentage of cell viability for varying TAP modified soft-liners.
Test used- Kruskal-Wallis test, triplicate sampling was carried out to assess the repeatability and to minimise the errors

Anti-inflammatory test: The results of the anti-inflammatory test, conducted using the egg albumin denaturation assay, revealed varying degrees of denaturation with TAP modified soft liners and Diclofenac sodium (standard drug) at different concentrations. At 10 µL, TAP modified soft liners exhibited 42% inhibition compared to 45% by Diclofenac sodium (standard drug). A similar pattern was observed at 20 µL, with TAP modified soft liners showing 53% inhibition compared to 57% by Diclofenac sodium (standard drug); at 30 µL, the values were 54% and 73%, respectively; at 40 µL, they were 72% and 78%, respectively; and at 50 µL, they were 77% and 81%, respectively. Diclofenac sodium consistently exhibited higher anti-inflammatory activity at all concentrations, while TAP modified soft liners showed lower inhibition of egg albumin denaturation across the concentration range [Table/Fig-10].

DISCUSSION

Soft liners with antimicrobial properties have not been extensively studied, as the majority of denture wearers have only reported fungal infections and denture sore spots. Dentists face difficulties in spot drug delivery systems, as the oral cavity is constantly swished with saliva, making drug delivery challenging unless the drug's substantivity is enhanced. These issues arise in cases like implant placement or bone augmentation procedures, where patients also prefer to be fully healed within two days of the procedure. For these patients, dentures modified with soft liners containing antibiotics can be provided. This study specifically focuses on the incorporation of TAP along with the soft liners, which are then coated onto the dentures [25,26].

Concentration of TAP	% of inhibition of TAP modified soft liners	% of inhibition of Diclofenac sodium (standard drug)
10 µL	42	45
20 µL	53	57
30 µL	54	73
40 µL	72	78
50 µL	77	81

[Table/Fig-10]: Anti-inflammatory analysis with varying concentrations of TAP-modified soft liners.

The addition of nanoparticles against fungi and spores is effective and popular for reducing denture sores and acting against *Candida albicans*. According to Chladek G et al., the addition of Ag Nps with the soft liners gave effective results, and the antifungal efficacy ranged from 16.3% to 52.5% [27]. In a similar manner, the addition of various antibacterial agents with commercially available soft liners is essential in situations immediately after intra-oral surgery, especially in cases where patients are periodontally compromised. Taylor RL et al., investigated the interaction between *Candida albicans* and different soft denture liner materials; unfortunately, none of the materials tested were effective in inhibiting candidal growth or preventing colonisation and penetration. This highlights the ongoing need for improved denture materials with antifungal properties [28]. For the antibacterial efficacy, Baygar T et al., in his study incorporated carvacrol, which reduced the adhesion of biofilm and also decreased the colonisation of plaque formation. A zone of inhibition of 41.33±1.53mm was reported for *Bacillus subtilis* and 32.33±0.58mm for *Streptococcus sanguis* [7].

The suture threads present in the oral cavity post-implant surgery can be susceptible to the microorganisms in the oral cavity. This susceptibility can be reduced by using antibiotics modified soft liners, which can enhance the healing of the implant site. In the study by Ansarifard E et al., Copper Oxide Nanoparticles inhibited the growth of *C. albicans* and oral *Streptococcus*. Similarly, in this study, there was a reduction in bacterial concentration assessed using bacterial plates [29].

In the present study, the addition of TAP has been effective against various bacterial strains such as *S. aureus, E. faecalis*, and *Pseudomonas*. The modified soft liners were also not toxic. Further studies are required to assess the clinical ability of the liners to evade the attachment of biofilms and to determine if the amount of bacterial load on the plaque is clinically reduced after using these soft liners post-surgery. Additionally, there is a need to assess the healing ability and anti-inflammatory efficacy of the modified soft liners.

Limitation(s)

Although TAP-modified soft liners show improved antibiotic, anti-inflammatory, and cytotoxic properties, further research with an improved sample size is needed to fully understand the therapeutic potential and safety profiles of these modified soft liners, including studies conducted on animals/patients.

CONCLUSION(S)

Incorporated into soft liners, triple-antibiotic paste has shown effective antibacterial management. It is recommended to use soft liners

with TAP for patients undergoing stage 1 implant surgery in long-span edentulous sites, where a removable denture will be provided as an interim prosthesis for a few weeks until the final prosthesis is fabricated. This is to effectively manage the postoperative bacterial load accumulation at the implant site. Further research is needed to determine the precise mechanism by which TAP influences the host's inflammatory response and to determine how well TAP can handle postoperative inflammation and bacterial load after implant surgery.

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