



Research Article

A COMPARATIVE STUDY TO EVALUATE THE ANTIFUNGAL EFFICACY OF NANO GRAPHENE OXIDE MATERIAL AND CLOTRIMAZOLE INCORPORATED IN SOFT LINERS IN COMPLETE DENTURES- AN IN VITRO STUDY

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ABSTRACT

Title: A comparative study to evaluate the antifungal efficacy of nano graphene oxide material and clotrimazole incorporated in soft liners in complete dentures- an in vitro study. **Methods:** 30 samples were prepared out of which 15 were coated with nano graphene oxide of 20 nm size and 5% solution combined with chloroform. The remaining 15 discs were incorporated with Clotrimazole. The above discs were placed on the flat bottom of well cell culture plates. Hundred microlitre of initial suspension in sabouraud's broth was inoculated to each well and incubated at 37 degree celsius. After incubation for 24 hours and 72 hours for extended contact period, suspension was withdrawn. Viable cells in the suspension were determined by using the spread plate method at the level of detection. The data obtained was subjected to statistical analysis. **Results:** Results of the study showed that, 1% Clotrimazole showed a larger zone of inhibition when compared to that of Graphene oxide combined with Chloroform. Thus, confirming the antifungal efficacy of soft liners containing graphene oxide and clotrimazole against candida species. **Conclusion:** Antifungal efficacy of 1% Clotrimazole was found to be better than Graphene oxide with Chloroform against Candida albicans.

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INTRODUCTION

Prosthetic rehabilitation of edentulous patients with complete denture is considered more than satisfactory by most patients due to their effects on mastication, phonetics and esthetics. Inevitably, there are certain post insertion problems like inability to chew, persistent pain and discomfort. To overcome these problems, soft denture lining materials have been used for decades. Soft denture liners are based on poly-methyl methacrylate or silicone elastomers. They are used to repair and rebuild the surface of the prosthesis in contact with the oral mucosa.¹

The soft liners can be helpful for a few days to a week but these materials should always be used as short-term materials. The Long-term soft liners are usually either a silicone or a plasticized acrylic. Plasticized acrylic materials usually have a shorter life span because of water sorption and hardening.² Thus, they have high percentage of plasticizers and composition similar to the denture base polymers. Therefore, Plasticizers keep the materials soft, but this quality is lost overtime. The acrylic soft liners may be used for up to a period of 6 months. Long-term silicone soft liners are could be used for up to 1 year. These liners have the great potential of improving the comfort of denture patients with ridge atrophy, non-resilient and thin mucosa, and bony undercuts.³ It has been reported that these soft liners have increased the satisfaction

level of denture wearers and Quality of life in terms of oral health is also improved. Owing to its poor physical properties in the oral environment, it promotes the colonization of microbes. The most common pathogenic organism is candida species. Microbial growth occurs by the adhesive interactions between candida species and oral streptococci.⁴ The denture stomatitis caused by candidiasis is a most common condition in complete denture wearers, characterized by generalized inflammation of the palatal mucosa covered by the denture. Candida encouraged denture-stomatitis can be managed by either repair of denture or replacement, prophylactic actions taken by the patients and prescribing antifungal drugs.⁵ Various antifungal agents have been incorporated in soft liners and have shown to cause significant increase in the material's hardness making it advantageous.⁶ Nano graphene oxide is known to have antimicrobial activity through surface contact. Graphene is a 2-dimensional carbon allotrope with the single layer of sp² bonded carbon atoms in the form of hexagonal configuration.⁷ Thus, It has closely packed honey-comb structure that contains various functional groups as epoxy, carbonyl, hydroxyl and carboxylic on surface and at the edges.⁸ Nano graphene oxides are considered to have potential antimicrobial activity through the surface contact with high physical and mechanical properties.⁷ Graphene oxide has shown outstanding potential in research fields, including biomedical applications and antifungal properties. Graphene

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oxide possesses several extraordinary physical, chemical, optical, electrical, and mechanical properties. Because of limited published clinical studies on nano graphene oxide testing its antifungal properties on soft liners, the present study stands unique to evaluate the fungal assay of candida albicans in soft liner material with incorporating nano graphene oxide and to evaluate the fungal assay of candida albicans in soft liner material with incorporating clotrimazole. The present study will also compare and evaluate the antifungal activity of nano graphene oxide combined with chloroform and clotrimazole in soft liner material.

MATERIALS AND METHODS

A total 30 discs were made of modeling wax of diameter 6mm and thickness 1mm in a denture acrylisation flask. The wax discs were invested on dental plaster after the first pour was set completely separating medium was applied to the first pour and second pour was mixed with plaster and dental stone and it was clamped. After the second pour is set completely the flask was dewaxed for 6 minutes and complete wax was eliminated and mould space for discs was created. 30 moulds were made, out of them the 15 moulds with soft liners mixed with 5% of nano graphene oxide that is 50 mg of graphene oxide in one gram of soft liner material. It was mixed in a homogeneous paste by continuous stirring on a glass slab and filled in all the 15 mould, these were considered as the study group (Group 1). In this group solvent were added for the Graphene oxide dispersion.

Group 1: Consist of 15 discs which were coated with nano graphene oxide of 20 nm size and 5% solution.

For breaking of graphene powder to nano graphene, First sonication of graphene oxide combined with Chloroform of 2 ml concentration was done using sonicator bath for about 30 minutes and then probe for 2 minutes. All the samples were subjected to dry in incubator so that solvents are evaporated little and solvents itself do not show any reaction. And these are mixed with 1 gm of soft liners.

Group 2: consist of 15 discs which were incorporated with 1% clotrimazole.



Figure 1 Sabouraud's dextrose agar plate showing zone of inhibition with clotrimazole incorporated in soft liners

The samples were cured at room temperature for 24hrs. Once the samples were completely cured discs were retrieved. The specimens were placed in sterile test tubes that contained sterile saline and specimens were cleaned in ultra-sonic unit with distilled water for 8 minutes to remove any kind of residues from their surfaces. Then specimens were all dried with absorbent paper. For susceptibility testing, a standard inoculum of yeast was used. The standard inoculums were prepared in the pure form to match the turbidity of $1.5 \times$

1.08 cfu/mL (equivalent to 0.5 McFarland) by transferring colonies of *Candida Albicans* to Sabouraud's Dextrose agar (100 ml) cultures and incubated for 24 hours.

Preparation of Yeast: To evaluate the antifungal effect of above mentioned two substances, A Lawn of *Candida albicans* fungi (PTCC5027) were prepared on Sabouraud's Dextrose Agar.



Figure 2 Sabouraud's dextrose agar plate showing zone of inhibition nano graphene oxide incorporated in soft liners

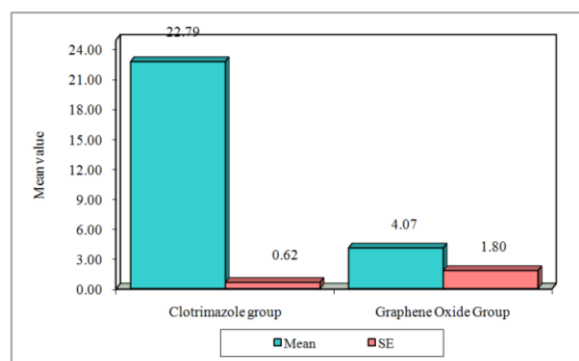


Figure 3 Comparison of two groups with Zone of Inhibition (in mm)

Microbiological Surveys: The 30 disinfected samples were divided into 2 groups of 15 discs each and then transferred into 6 different plates (5 discs in each plate). Sabouraud Dextrose agar (SDA) medium 100 ml was used to investigate the presence of *Candida albicans* fungi. Using a sterile tweezer, 30 of the disinfected discs were spread on each of the 6 plates harboring *Candida Albicans*. Above samples were placed on the flat bottom of well cell culture plates, and 100 microlitre of initial suspension in 1ml of sabouraud broth was inoculated to each well and incubated at 37 degree celsius. After incubation for 24 hours and 72 hours for extended contact period, suspension was withdrawn, viable cells in the suspension was determined by using the spread plate method at the level of detection. The obtained data was subjected to statistical analysis and the results were tabulated.

RESULTS

The present study was designed to evaluate the antifungal efficacy of nano graphene oxide and clotrimazole incorporated in soft liners in complete dentures. The antifungal effect with nano graphene oxide material and clotrimazole against candida species were demonstrated by Disc Diffusion Test after 24 hours of incubation time. Total 30 samples were used for this study 15 discs were incorporated with nano graphene oxide material and remaining 15 discs were incorporated with 1% of clotrimazole. The results of the study reveals that discs incorporated with both types of antimicrobial agents showed

zone of inhibition. (figure 1 and figure 2). The data obtained from the results of the study was subjected to statistical analysis (figure 3).

1. Soft liners of complete dentures modified with Clotrimazole and nano graphene oxide, both the groups showed effective disinfection against *Candida albicans*.
2. On analysis of Disc Diffusion Test, Clotrimazole showed significantly more reduction of candida colonies than that of nano graphene oxide, clearly indicating its anti-fungal nature.
3. The study concluded that 1% Clotrimazole was found to have a better zone of inhibition than nano graphene oxide of 20 nm size against the *Candida albicans*.

Table 1 Summary of Zone of Inhibition (in mm) in two groups

Groups	Mean	SD	SE	Median	Quartile	Range
Clotrimazole group	22.792	2.330	0.622	3.003	0.003	0.00
Graphene Oxide group	4.076	0.981	0.800	0.001	0.015	0.00

Table 2 Comparison of two groups with Zone of Inhibition (in mm) by

Mann-Whitney U test

Groups	Mean	SD	Median	Mean rank	U-value	Z-value	P-value
Clotrimazole group	22.792	2.332	3.002	2.50			
Graphene Oxide group	4.076	0.980	0.008	0.00			

*p<0.05

DISCUSSION

Denture stomatitis is commonly seen in old denture wearer patients and it is multifactorial in origin. It can cause by tissue trauma from poorly maintained denture hygiene, ill-fitting dentures, continuous denture use without removal, xerostomia, and compromised medical condition.⁹ An important feature of *Candida albicans* in causing infection is their ability to adhere and form biofilms on host surfaces. Since *Candida* is an opportunistic organism, it has developed different resistant mechanisms due to its discriminated use of antifungal agents. Moreover, it is difficult to kill these organisms owing to their survival characteristics in the host. So more specific antifungal therapies has been the focus of research.¹⁰

Okita *et al* (1991) stated that the tissue conditioners had more microbial colonization compared to acrylic resin.¹¹ A study was conducted in 1994 in which antifungal agents; nystatin and Clotrimazole were incorporated in to silicone sample at 1%, 2.5%, 5% concentration by weight. Hence, it was observed that the frequent washing will not diminish the amount of clotrimazole which were added to a silicone soft liner material for clinical use. This study concluded that Clotrimazole was more effective in inhibiting the growth of the fungus than nystatin when tested by disk diffusion method.¹² Another study was conducted in 2009 to assess the inhibition of the growth of *C. albicans* when incorporating clotrimazole. It was concluded that addition of clotrimazole significantly reduced *C. albicans* growth on the surface of the silicone soft liner and samples also continued to inhibit the fungal growth when they were washed daily with wet cotton for 2 months.¹³ Pachava K *et al.* in their study in 2014 showed the similar results and stated that antifungal agents are

considered potent and safe for both topical and systemic mucosal lesions.¹⁰

Correspondingly these medicines have been widely targeted for incorporation into soft liners and outcome was acceptable for the inhibition of candidal growth. In addition to nystatin, azole group derivative is being tested. These agents are considered to be potent antifungals and are of comparable efficacy to nystatin.^{9,14} The effective duration and stability of added antifungal agents remain between 3 and 7 days or maximum for 14 days.¹⁵

Graphene is one of the known antimicrobial agents. Di Giulio M *et al* (2018) assess the antibacterial activity for both gram Gram-negative and Gram-positive and microbes. This study was done to evaluate the antifungal activity of pathogens that includes *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. It was stated that graphene oxide shows antibiofilm efficacy and antimicrobial against chronic wound microorganisms that too include *Candida albicans*.¹⁶ The wrapping and photothermal ablation mechanism also cause the cell damage.¹⁷ The acrylic-based tissue conditioners (GC Soft liner) alone do not show any antifungal activity.¹⁸ The minimum inhibitory concentration of graphene oxide against to nosocomial pathogens is proved to be in the range of 2.5 to 5%.¹⁹ Rayannavar S *et al* (2020) have proved that antifungal activity was enhanced by increasing the concentration up to 5% of nGO. The results showed that the diameter of inhibition zone (DIZ) was maximum when 5% nano graphene oxide was added for both 1 day and 7 days after with the mean values of 17.2mm and 14.4mm respectively.⁷ Kanathila H *et al* (2011) showed the similar results.¹⁸ A study done by Lee JH *et al* (2018) concluded that 2% nGO incorporated in PMMA showed constant antimicrobial adhesive property against *Candida albicans* for about 28 days. Results showed that PMMA presented better antimicrobial-adhesion effects after incorporating graphene oxide.²⁰ Gamal R *et al* (2019) proved that 0.05% nGO when added with PMMA inhibits the growth of *Streptococcus mutans* microbes.⁸ Azevedo L *et al* did a study in 2019 by using an intraoral digital impression and a 3-dimensional facial scan of patient. Follow-up evaluations were made at 1 week and 1, 3 and 8 months. The incorporation of GO in polymethyl methacrylate resins seems to be a good option for prosthetic rehabilitation and provide a reliable, definitive material option.²¹

From the present study it was demonstrated that soft liners incorporated with graphene oxide of size 20nm size and 5% solution combined with Chloroform of 2 ml concentration can exhibit antimicrobial behaviour against candida species. But Clotrimazole shows more antimicrobial behaviour than that of graphene oxide. Thus, confirming graphene oxide as an alternative antifungal therapy to antibiotics. In this study, it was observed that Clotrimazole has the best inhibition against *Candida albicans* when compared to graphene oxide. The most important feature of graphene oxide is that it does not make bacteria resistant to disinfectant materials, highly biocompatible and does not induce any allergic reactions.

Clinical Implications

Soft liners of dentures are in contact with saliva and thus they are inevitably susceptible to bacterial colonization which later causes subsequent degradation of the material and infection of

surrounding tissues. By increasing the concentration of Nano Graphene oxide (nGO) shows significantly increased antifungal activity. Thus, nGO can be used to reduce the fungal growth and has good potential to treat candida induced denture stomatitis by drug delivery with soft liners. To overcome the above drawback, use of Nano Graphene oxide and Clotrimazole with antifungal properties in conjunction with soft liner of dentures offers promising results.

CONCLUSION

The following are the conclusions from this study:

1. Within the limitations of this study, it was concluded that soft liners of complete dentures modified with Clotrimazole, nano graphene oxide both the groups showed effective disinfection against *Candida albicans*.
2. On analysis of Disc Diffusion Test, Clotrimazole showed significantly more reduction of candida colonies than that of nano graphene oxide, clearly indicating its anti-fungal nature.
3. The study concluded that 1% Clotrimazole was found to have a better zone of inhibition than nano graphene oxide of 20 nm size against the *Candida albicans*. Further investigations regarding modified soft liners with nano graphene oxide with the solvents are necessary to find ways to improve its long term clinical efficacy and physical behavior.

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