Section A-Research paper
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Preparation, Characterization and Biological Effect of Chitosan Conjugated Linezolid Combined with the Liquid of Glass Ionomer Cement for Improved Dental Restorations

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Abstract:

Background: The management of dental caries revolves around prevention, control and removal of caries. Even after mechanical removal of caries, some part of infected dentin with viable bacteria remains hidden to the eyes of the clinician.Further, fluoride release of GIC is not sufficient to control the bacteria after loner clinical service. There is a need to device an effective material that had all advantages of GIC with additional antibacterial action.

Aims: To evaluate "Linezolid loaded chitosan" nanoparticles, added to GIC and test for its physic(o)chemical and in vitro biological properties.

Materials and Methods: Chitosan nanoparticles were synthesized and loaded with linezolid. Six different groups of GIC were prepared of which one was control group, that was devoid of any modifications. Five test groups were prepared by modifying the liquid. The GIC fluid was modified with 1, 2, 3, 4 and 5 wt% CH. Characterization was done by FTIR spectra, HPLC for release profile, Antibacterial activity studies using 3 bacteria and Invitro Cytotoxicity assays using human gingival fibroblast cells. Live/Dead assay was performed.

Results: FTIR spectra showed successful incorporation of linezolid in chitosan. HPLC studies showed huge burst of release on the first day and lower amounts releasing on the 7th day.

Antibacterial activity was present in all groups. Cytotoxicity assays showed that all modifications are biocompatible. and the preparation has great potential for use in the clinical scenario.

Conclusion:

The hypothesis that Linezolid, delivered through chitosan can offer prolonged antibacterial activity in the clinical service of GIC is accepted within the limitations of the study.
Keywords: GIC, Linezolid, Antibacterial, Cytotoxicity, Nanoparticle

Introduction

The polymicrobial infectious disease of Dental Caries is known from antiquity and is still a public health concern.(1) The management of dental caries revolves around prevention, control and removal of caries. Prevention takes various forms like topical fluoride application, prophylactic pit and fissure sealants etc. Control involves caries control restoration while removal of caries refers to complete cavity preparation for restoration of teeth. Further, atraumatic restoration also belongs to caries removal category, where GIC is extensively used. The adhesive nature and fluoride release of GIC are two critical properties of GIC that makes the cement a well sought after one.

However, even after mechanical removal of caries, some part of infected dentin would definitely remain, hidden to the eyes of the clinician. In other words, cariogenic bacteria can still remain after cavity preparation, in both ART and Conventional cavities.(2) Though it was initially opined that fluoride release of GIC can inhibit these bacteria, it is reported that in the longer clinical service of GIC, it is not sufficient to control the bacteria.(3)

In order to prevent these bacteria from causing secondary caries, it is essential to control them using effective antibiotics. Therefore, there is a search for GIC cement with effective antibacterial properties. Ions that travel in and out of GIC have inspired the investigators to add antibiotics that can diffuse out of GIC into the dentin. (4) Various antimicrobial agents tried include chlorhexidine, doxycycline, metronidazole, ciprofloxacin, cefaclor, cetrimide, minocycline and so on. They have shown good results but have threatened the physicochemical properties of GIC at useful concentrations.(2)

Therefore there is a need to device an effective material that had all advantages of GIC with additional antibacterial action.

Linezolid (LZ) is one of the oldest drugs in the oxazolidinone antibiotics category, approved by the US FDA in 2000. It is known to act by preventing the synthesis of bacterial protein via binding to rRNA on both the 30S and 50S ribosomal subunits. It is used to treat various antibiotic resistant bacterial infections.(5) The drug cannot be dispersed in the cement as it may lose its properties in the chemical reaction, requiring a suitable host to carry, retain and release them in the clinical service of GIC.

Chitosan(CH) is a biocompatible polymer derived from the chitin found in crustacean shells. It is widely used in various formulations and biomaterials.(6) Chitosan is known to possess mild

antibacterial properties and is reported to improve the mechanical properties of glass ionomer cement. (7) Therefore, it can serve as a suitable host for the said purpose.

Hypothetically, antibiotic Linezolid, delivered through chitosan can offer prolonged antibacterial activity in the clinical service of GIC. There is a lacuna in the literature with regard to delivery of linezolid to GIC by loading it in nanochitosan. Therefore in this study, "Linezolid loaded chitosan" nanoparticles are added to GIC and tested for its physic(o)chemical and in vitro biological properties.

Materials and Methods:

Chitosan and Sodium tripolyphosphate were purchased from Sisco Research Laboratories pvt limited. India and used to prepare nanoparticles. Acetic Acid was purchased from Merck. These chemicals were used as received.

Synthesis of Chitosan nanoparticles

Chitosan(CH) nanoparticles were prepared by previously reported procedures using ionotropic gelation technique.(8,9) Briefly, Sodium tripolyphosphate (0.4 wt.%) was added to aqueous Chitosan solution. Foe every 2.5gm of chitosan, 1 gm of Sodium tripolyphosphate was used. After vigorous stirring for 40 min, chitosan nanoparticles were formed. Antibacterial loaded Chitosan nanoparticles were fabricated by the incorporation of appropriate quantities of antibiotics into the chitosan solution prior to mixing of TPP solution. Thus formed nanoparticles centrifuged at 9000 rpm for 30 min at 4°C and collected, washed with double distilled water, again suspended in distilled water for ultrasonication for 30 s for obtaining a homogenous suspension. The solution was freeze-dried to obtain the nanoparticles.

Incorporation to GIC Liquid and sample preparation

Six different groups of GIC were prepared of which one was control group, that was devoid of any modifications. Five test groups were prepared by modifying the liquid. The GIC fluid was modified with 1, 2, 3, 4 and 5 wt% CH. Subsequent to addition of chitosan, the GIC liquid was stirred magnetically for 1 hour and incubated at 37 °C for 24 h. Then, for fabricating cement samples, liquid and powder were mixed according to the manufacturer's instructions (powder/liquid ratio of 3.6/1.0 g). (10)

Characterization by Fourier-transform infrared spectroscopy

In order to confirm the chemical composition of GICs, samples were processed for obtaining FTIR spectra using conventional potassium bromide pellet technique (1:100 ratio). FTIR spectra was recorded using Bruker alpha 2 FTIR-ATR spectrometer and FTIR spectrum was recorded from 400 to 4000cm^{-1} with a resolution of 4 cm^{-1}.

High Performance Liquid Chromotography for release profile

Samples were stored in phosphated buffered saline at 37^{o}C under sterile conditions for specified periods of time, viz. 1 and 7 days. At the specified duration, the buffer was presented for HPLC analysis to detect the presence of linezolid. HPLC analysis was performed using WATERS - 2695 HPLC system. It consisted of auto-sampler, quaternary pumps, column oven and UV detector. Data was analysed using Empower-3 software. Separation was done using C18 column (Shim pack, 250 mm X 4.6 mm, 5 µm) with mobile phase consisting of methanol: water (80:20, v/v) at the flow rate of 1.0 ml/min. Detection was performed at 256 nm. Column was equilibrated at 25 ^{0}C. Injection volume was 20 µl. The method was carried out for each sample at 1st day and 7th day. The area under the curve was used to calculate the concentration of the drug.

Antibacterial activity studies

Antibacterial activity of samples was tested against Streptococcus mutans, Lactobacillus acidophilus and Actinomyces viscosis. Mueller Hinton agar was used for S. Mutans and L. Acidophilus, while Brain heart infusion agar was used for Actinomyces viscosis in anaerobic condition. Initially a lawn culture was prepared and wells were cut using sterile glass tube in the agar plate. Freshly mixed cements were placed in the wells and left to incubate for 24 hours at 37 ^{o}C. Inhibition zones around the specimens were subsequently measured.(11) Three specimens were tested for each group.

Invitro Cytotoxicity assays using human gingival fibroblast cells

Human Gingival Fibroblasts Cells were purchased from ATCC (PCS-201-018). Cells were cultured with RPMI 1640 (Invitrogen Corporation, CA, USA) supplemented with 20% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C with 5% CO_2. The culture medium was changed every three days and sub-cultured at 80% confluence. At passage 2, cells were used for all *in vitro* experiments for this study.

Cell Viability/ MTT assay:

Elution method was used to determine the cytotoxicity of GIC samples. Sterile Samples from all 6 groups were placed in cell culture media for 24 hrs to allow the elution of soluble substances from samples. The medium was retrieved and used for MTT assay.(12) HGF cells were seeded on 96well culture plate and cultured in elution media for 24hr and 48hrs. To determine percent viability, the post incubated cells were analysed by standard MTT assay. Another set of wells were seeded similarly for live/dead assay.

Live/Dead Assay

Live/Dead Viability/Cytotoxicity kit (Calcein-AM dye, Invitrogen, USA) was used as per the manufacturer's instructions. Cells seeded and grown with elution media were stained after 24 hours and observed using inverted Phase contrast fluorescence microscopy (Invitrogen, evos). Viable cells, exhibiting green fluorescence, were stained by Calcein-AM only. Live and dead stained cells were counted manually and a ratio of live to dead cells was calculated for each well.

Statistical Analysis:

Statistical analysis was carried out with R statistical package (4.1.1). Descriptive statistics was given by mean and standard deviation. Antibacterial activity and viability was tested by ANOVA with post hoc tukey test. p value less than 0.05 was considered significant.

Results

Fourier-transform infrared spectroscopy:

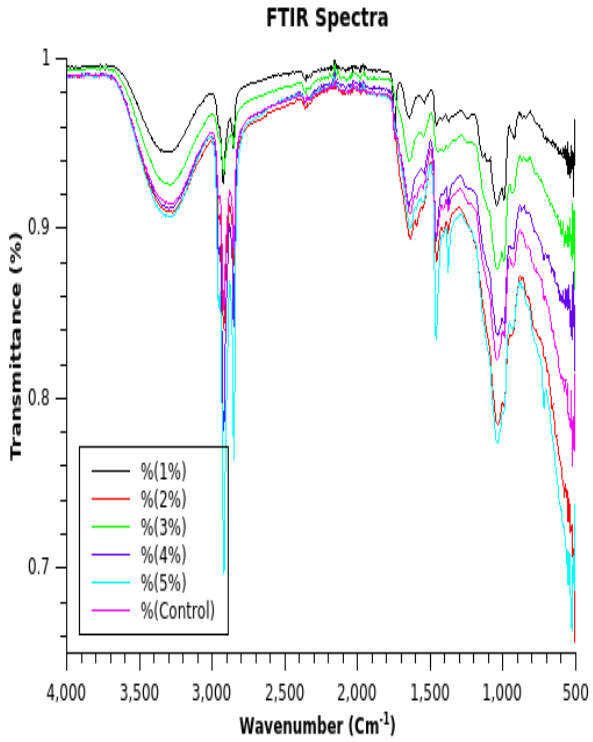

Fig. 1. FTIR Spectroscopy

FTIR spectra showed successful incorporation of linezolid in chitosan, as superimposed spectra of both the molecules were clearly seen

HPLC (High Performance Liquid Chromotography

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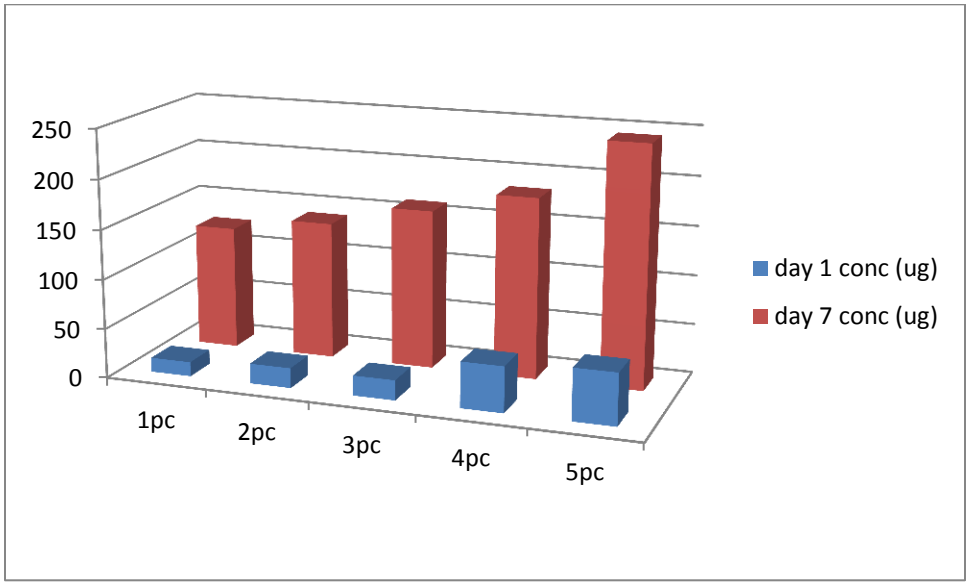

Fig. 2. HPLC Analysis

The release studies done using HPLC showed a huge burst of release on the first day and lower amounts releasing on the 7th day. Therefore, the release is proceeding till or beyond 7th day.

Antibacterial activity studies

Table 1. Mean zone of inhibition

	1pc	**2pc**	**3pc**	**4pc**	**5pc**	**Control (0%)**	**p Value**	
Strep mutans	13±1	13±1	15±0	15±1	19±1	21±1	2.72E-08	Sig
Lactobacillus acidophyllus	12±3	13±1	14±2	14±2	15±1	10±0	0.0174282	Sig
Actinoomyces viscosus	13±1	14±1	15±1	16±0	16±1	17±0	4.72E-06	Sig

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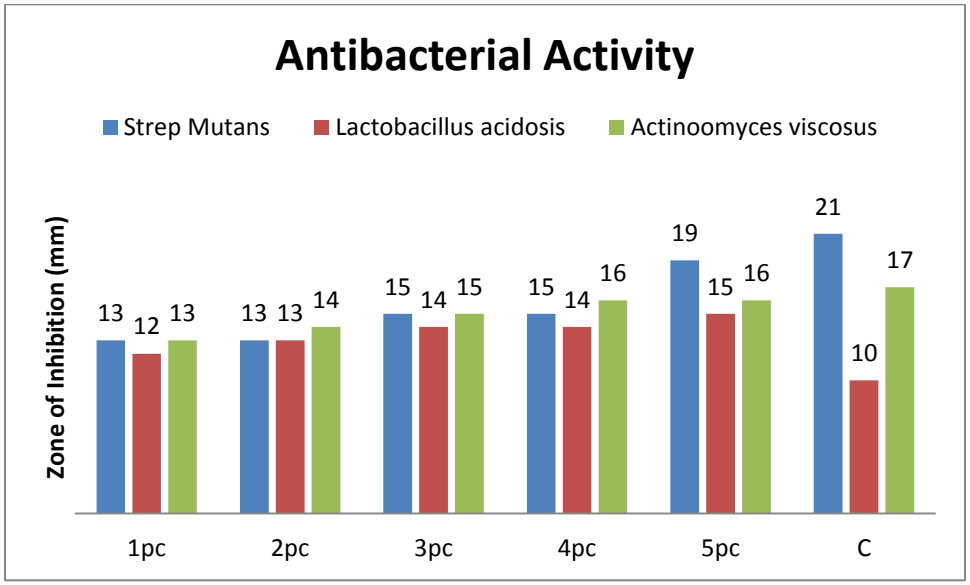

Fig. 3. Antimicrobial activity

Form the results of antimicrobial studies, it was seen that control GIC had the best inhibition potential against S. mutans and A. viscosus. Addition of chitosan produced significant change in antibacterial activity against L. acidosis. However, it retained its antibacterial activity against other two species after addition of nanoparticles.

Cytotoxicity Assay

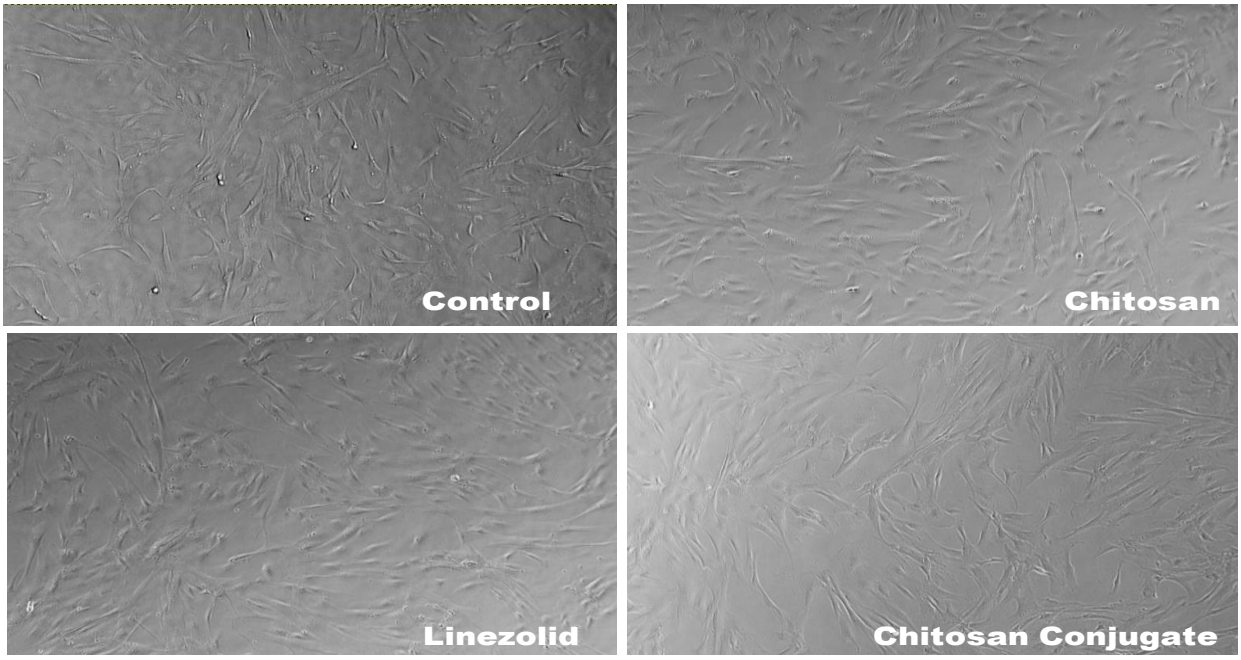

Fig. 4. Images of cells treated with chitosan, linezolid and chitosan conjugate

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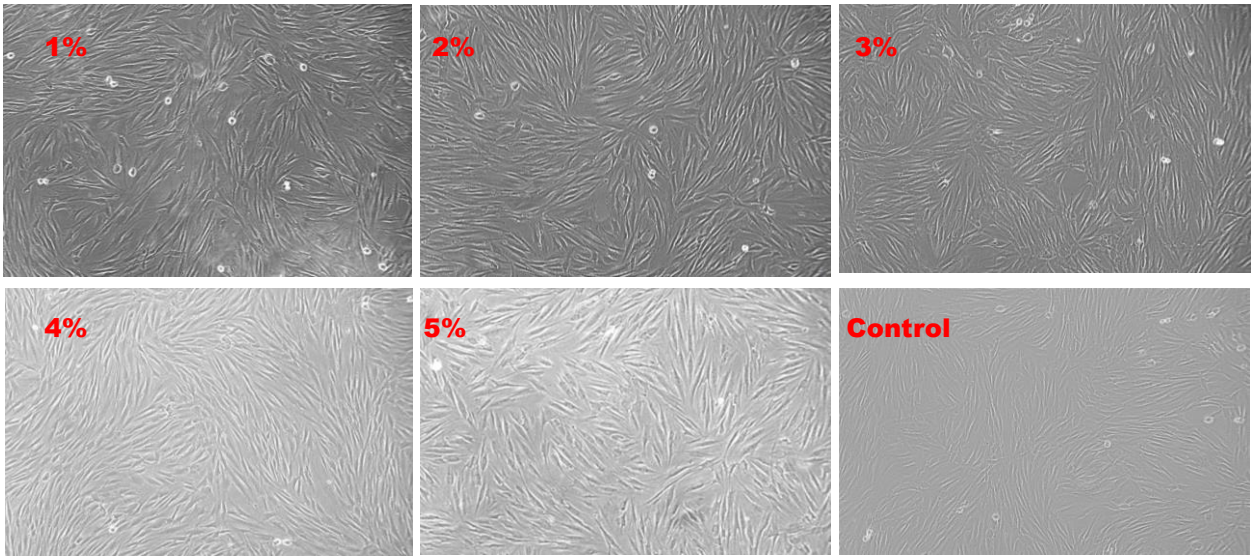

Fig. 5. Images of cells treated with various concentrations of linezolid

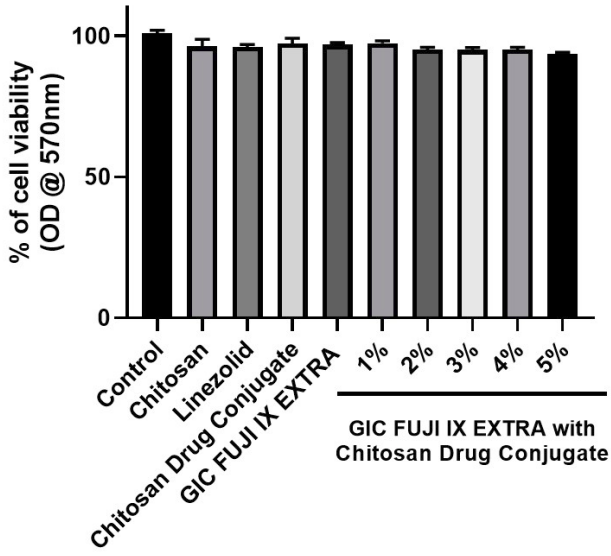

Fig. 6. Cell viability Assay

With regard to the in vitro biocompatibility analysis, the results showed statistically significant difference between the test and control. As observed, the viability increased with concentration and chitosan also aided in proliferation of cells. It shows that all groups were better than control except for 5% group, which showed less viability than control. However, at 48 hours all groups were better than control cement. Hence, it can be inferred that all modifications are biocompatible and the preparation has great potential for use in the clinical scenario.

Live Dead Assay:

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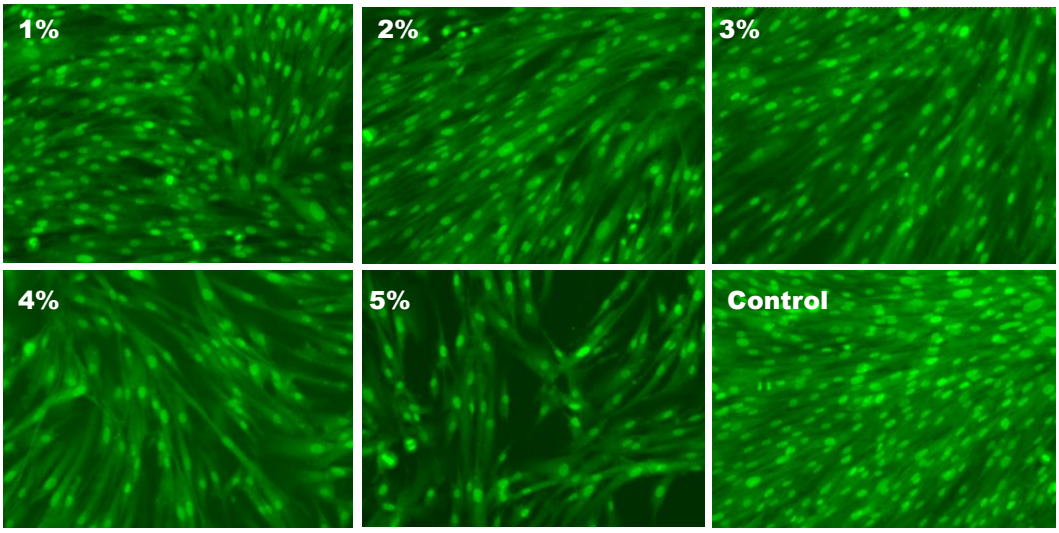

Fig. 7. Live Dead Assay – Florescence microscopy Images

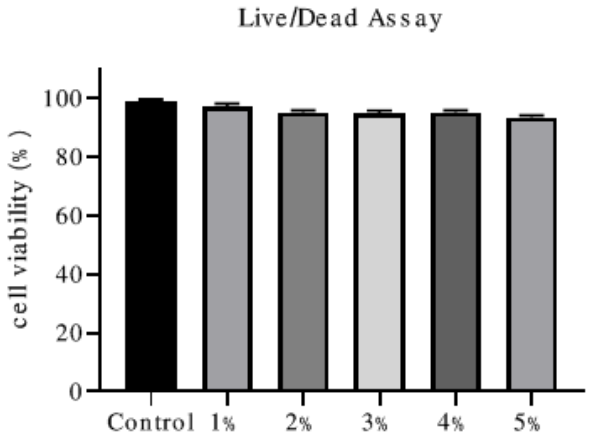

Fig. 8. Quantitative analysis of cell viability from live/dead assay

Discussion

The most extreme example of conservative dentistry is deep caries restoration, which is frequently difficult in terms of complete caries removal. There is currently a clinical need for an antibacterial restorative material that can effectively prevent secondary dental caries. Glass ionomers have been used to control caries in a variety of ways, Class III, Class V, and caries control restorations, as well as atraumatic restorative treatment. However, residual bacteria left over from caries removal can cause secondary caries. Though the fluoride content of GIC has some inherent antibacterial activity, it is insufficient for the restoration to provide longer clinical service. Antimicrobial agents added to GIC have thus been researched in the literature to prevent secondary caries. However, the addition of such agents has a negative impact on the material's physicochemical properties. In order to retain the advantageous properties of GIC and to increase the antibacterial effect, chitosan has been used to modify GIC. It has previously been shown that

it has enhanced various properties of GIC. In current context, increase in antibacterial properties by addition of various concentrations of Linezolid is studied by FTIR, release profile by HPLC, antimicrobial and in vitro biocompatibility are studied.

Chitosan's FTIR spectrum is expected to reflect the functional groups present in chitosan in the major way. Peaks at 564 cm^{-1} (out-of-plane bending NH, out-of-plane bending C–O), 711 cm–1 (out-of-plane bending NH), 1604, 1598, and 1592 cm^{-1} (Vibrational mode of amide C═O stretching), 1174 cm^{-1} (C–O–C stretching), 2865 cm^{-1} (CH2 stretching), and 3594 cm^{-1} (−OH stretching) are characteristic of chitosan. Peaks at 2930 and 2830 cm^{-1} are characteristic of aliphatic chains. A broad peak beyond 3000 cm^{-1} is the hydroxyl group and moisture content. The spectrum was comparable to that of previous investigators.(13) Addition of linezolid, resulted in interaction with chitosan spectrum and superimposition. According to Reddy et al., (2013)(14) IR peaks of Linezolid includes 3338 attributed to N-H stretching, 3117, 3066 from aromatic C-H stretching, 2971, 2863, 2818 from aliphatic C-H stretching, 1738, 1662 from C=O stretching, 1545, 1516, 1453 from aromatic C=C stretching, 1425 from C-N stretching, 1381 from aliphatic C-H bending, 1334 from C-F stretching, 1274 from C-O stretching, 1198, 1177 from C-N bending, 1117, 1081 from aromatic C-H bending. Major peaks of linezolid according to Nidam et al., (2006)(15)include 1743, 1644, 1519, 1235 and 1117 cm-1. Of these, most of them superimposed with chitosan, resulting in more pronounced expression, rather than individual expression. Therefore, there is an interaction between chitosan and linezolid.

HPLC analysis is made to detect the presence of linezolid in buffer solution after 1 and 7 days. The linezolid incorporated in chitosan, which in turn incorporated in GIC, is intended to release in the buffer after setting. This is the basic premise of testing the antibacterial activity of cement. Estimation of drugs in buffer is effectively done by HPLC as it is accurate, reliable and reproducible. It is a commonly used technique for the purpose. In this study, HPLC analysis has shown release of linezolid from the set cement effectively in both time and dose dependant way. Increase in dose or concentration has led to increased release and increase in duration has also shown increased concentration in buffer. Previous studies have studies release of drugs from GIC. Kim et al., (2016)(16) have studied release of chlorhexidine from GIC and have shown initial burst of release and slowdown later. Yesilyurt et al., (2009)(11) have studied the release of ciprofloxacin, metronidazole and minocycline from GIC. Their study also showed increase of release after a week in buffer. Incorporation of linezolid into chitosan before incorporating into GIC has not practically shown any difference in drug release.

With regard to antimicrobial property of modified GICs, there was a statistically significant and dose dependant increase in antibacterial activity against all pathogens tested. As shown by Yesilyurt et al., (2009)(11) unset specimens have higher antibacterial activity and hence was analysed in the current study. Similar to their study, increase in concentration of drug showed increased antimicrobial activity as a converse of drug release. Similar scenario was reported by Kim et al., (2016). Therefore, release profile has reflected itself in antimicrobial activity also.

Ersahan et al., (2020)(17) tested the biocompatibility of various commercially available GICs on mouse fibroblasts and human dental pulp cells and found that all of them showed more than 100% viability compared to control, except one brand. Further, GICs inherently have lower cytotoxicity when compared with other biomaterials (18). Previous studies have shown increase in cell proliferative activity with addition of chitosan (19). However, presence of linezolid can modify these reactions and hence MTT assay was performed. In vitro biocompatibility studies showed good biocompatibility of various GICs used in the study. The result is comparable to conventional GIC. In fact, In the clinical perspective, all concentrations of linezolid showed higher viability than control. Further, Live-Dead assay performed on second day showed that all groups had excellent cell survival comparable to control group. Clinically all test groups showed that viable cells were over 90%.

Conclusion

The hypothesis that Linezolid, delivered through chitosan can offer prolonged antibacterial activity in the clinical service of GIC is accepted within the limitations of the study. Therefore the material proposed can be taken for further analysis leading to clinical translation. While all properties increased with increase in nanoparticle (CH-LZ) concentration, biocompatibility analysis showed 3% as optimal concentration.

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