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Original Article

Microbiology Section

Antimicrobial Activity and Stability of Electron Beam Irradiated Dental Irrigants

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ABSTRACT

Background: The electron beam (e-beam) radiation is considered as an effective means of sterilization of healthcare products as well as to induce the structural changes in the pharmaceutical agents/drug molecules. In addition to structural changes of pharmaceutical it also induces the formation of low molecular weight compounds with altered microbiological, physicochemical and toxicological properties. Among the several known medicaments, sodium hypochlorite (NaOCl) and chlorhexidine digluconate (CHX) are used as irrigants in dentistry to kill the pathogenic microorganisms like *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus mutans* and *Candida albicans* inhabiting the oral cavity.

Objectives: The aim of this study was to evaluate the antimicrobial activity and stability of e-beam irradiated dental irrigants, NaOCl and CHX.

Materials and Methods: Two dental irrigants NaOCl (1.25% and 2.5%) and CHX (1% and 2%) were exposed to various doses of e-beam radiation. The antimicrobial activities of e-beam irradiated irrigants were compared with the non-irradiated (control) irrigants against *E. faecalis*, *S. aureus*, *S. mutans* and *C. albicans* by disc diffusion method. Following the storage, physico-chemical properties of the irrigants were recorded and the cytotoxic effect was evaluated on human gingival fibroblast cells.

Result: The irrigants, 1.25% NaOCl and 1% CHX showed significantly increased antimicrobial activity against both *E. faecalis*, (16±0.0) and *S. aureus* (25±0.0) after irradiation with 1 kGy e-beam. Whereas, 2.5% NaOCl and 2% CHX showed slightly increased antimicrobial activity only against *S. aureus* (28±0.0). The significant difference was noticed in the antimicrobial activity and cytotoxicity of irradiated and non-irradiated irrigants following the storage for 180 d at 4^{0}C.

Conclusion: The e-beam irradiation increased the antimicrobial activity of irrigants without altering the biocompatibility.

Keywords: Antimicrobial activity, Cytotoxicity, Dental irrigants, Electron beam irradiation

INTRODUCTION

Radiation sterilization has been accepted as an ideal means of sterilization of healthcare products worldwide. Despite of their remarkable beneficial effect, the main concern of using ionizing radiation for sterilization of pharmaceuticals is the risk of formation of radiolysis products with altered structure, physicochemical, microbiological, and toxicological properties. It has been reported that 10 kGy gamma irradiation did not alter the antimicrobial activity of sodium ampicillin and penicillin G plus procaine [1]. The dosages of 15 kGy and 30 kGy of gamma and electron beam radiation did not affect the structural stability and antimicrobial property of chloroamphenicol, respectively [2]. Ionising radiation has been proved to be an effective technology to decompose organic substances and reduce the toxicity [3]. Several studies have been reported on the application of gamma or e-beam irradiations for sterilization and to evaluate the physico-chemical properties of antibiotics, however, no studies have published on the use of gamma or e-beam irradiations and to evaluate their effects on dental medicaments.

It has long been considered that NaOCl and CHX are sporicidal, vermicidal and wide spectrum antimicrobial compounds that kill the organisms by damaging their intact cell wall [4-6]. Furthermore, the studies have also been reported the strong cytotoxic effect of commonly used disinfectants in dentistry, NaOCl and CHX on the periradicular tissues [7,8]. Chlorhexidine digluconate by virtue of its broad spectrum antimicrobial activity, destroy bacteria by acting on their cell membrane [9]. However, the topical applications of chlorhexidine induce the anaphylactic reactions [10,11].

Among the oral pathogens, *E. faecalis* and *S. aureus* are the predominant bacteria responsible for 30-70% of root canal failures [12] and infections in oral region such as angular cheilitis, some endodontic infections, osteomyelitis of jaw and parotitis, respectively [13]. *S. mutans*, generally found in human dental plaque, has been known to cause tooth decay in humans [14]. *C. albicans*, the most common opportunistic pathogen in immunocompromised patients [15] is responsible for oral candidiasis associated with the denture stomatitis seen in 65% of denture wearers [16]. The currently used medicaments in oral treatments seem to be inefficient in complete removal of the pathogens. Hence, this study was carried out to understand the possibility of increasing the antimicrobial activity of two common dental irrigants, NaOCl (2.5% and 1.25%) and CHX (2% and 1%) treated with e-beam irradiation.

MATERIALS AND METHODS

Microorganisms: The type strains of *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), *Streptococcus mutans* (MTCC 890) and *Candida albicans* were obtained from Central Research Laboratory, Nitte University. The Stock cultures of these strains were maintained at -80^{0}C.

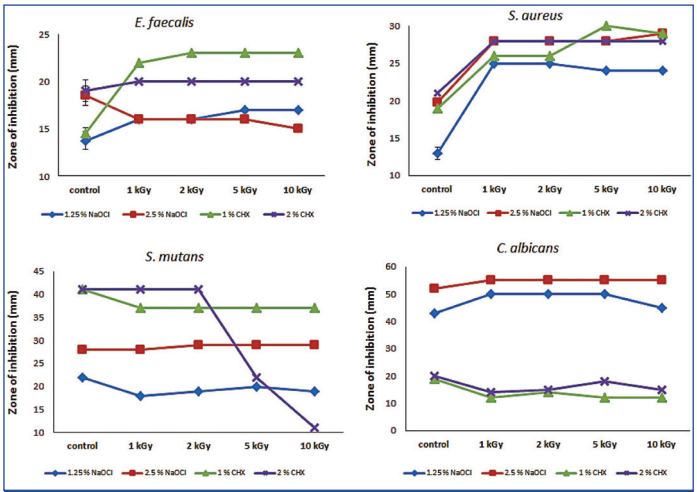
Cell line and maintenance: Human Gingival Fibroblast cell line was kindly obtained from Manipal Life Sciences Center, Manipal. The cell line was subcultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine Serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml of amphotercin B at 37^{0}C and 5% CO_2 in humidified incubator.

Irrigants: Sodium hypochlorite was prepared at 2.5% and 1.25% concentrations by diluting 4% NaOCl (MERCK) and chlorhexidine digluconate were prepared at 2% and 1% concentrations by diluting 20% chlorhexidine digluconate (SIGMA) in sterile double distilled water. Similarly prepared non irradiated irrigants were served as controls.

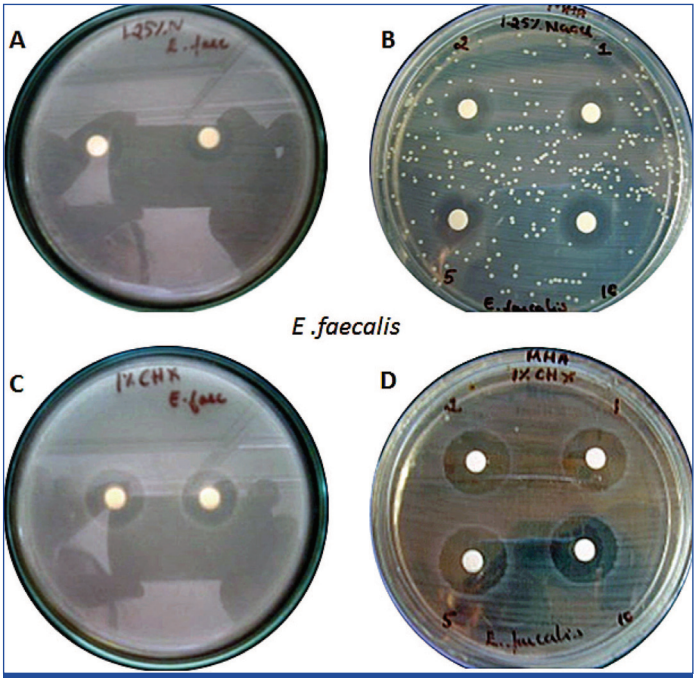
Electron Beam Irradiation of Irrigants: The radiation facility available at Microtron Centre, Mangalore University, designed by Centre for Advanced Technology, India was used for irradiation of irrigants (NaOCl and CHX). The irrigants (10ml) filled sterile polythene pouches were subjected to 1 kGy, 2 kGy, 5 kGy and 10 kGy of e-beam irradiation at a dose rate of 500 Gy/min. The irradiated

Groups	Irrigants	Day 1	Day 180
Control	1.25% NaOCl	9	9
	1% CHX	8	8
Irradiated with 1 kGy	1.25% NaOCl	9	9
	1% CHX	8	8

[Table/Fig-1]: pH of irrigants on day 1 and day 180



[Table/Fig-2]: The antimicrobial activity of irrigants before and after e- beam irradiation

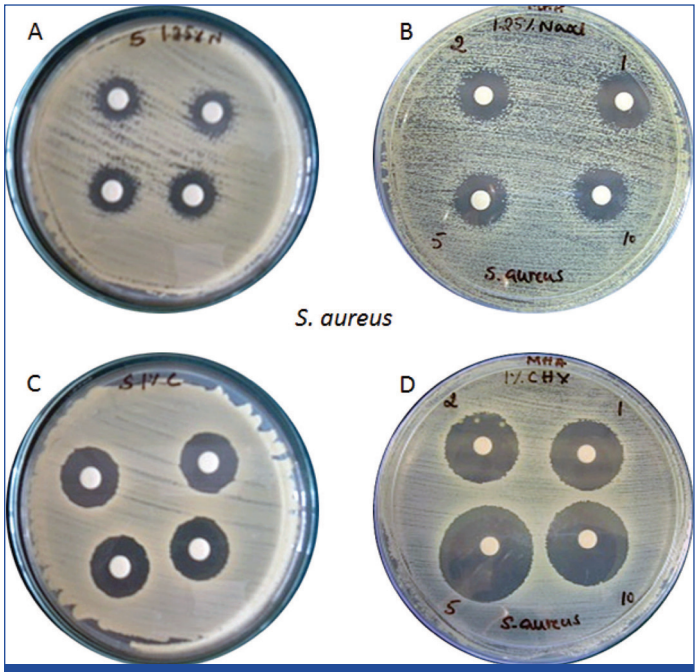


[Table/Fig-3]: The zone of inhibition by 1.25% NaOCl and 1% CHX against *E. faecalis*, A: Control 1.25% NaOCl; B: Irradiated 1.25% NaOCl; C:Control 1% CHX; D: Irradiated 1% CHX

as well as control irrigants were evaluated for their antimicrobial, physico-chemical and cytotoxic effect on day 1 and then stored in sterile vials at 4°C for 180 d.

Physico-chemical parameters: The colour, sedimentation, precipitation of control and irradiated irrigants were noticed on day 1 and day 180. The pH of irrigants was recorded using pH strips (Fisher Scientific) of range 2 to 10.

Antimicrobial susceptibility assay: The antimicrobial activity of the irrigants was determined by modified Kirby-Bauer [17] disc diffusion method. The suspension culture of *E. faecalis*, *S. aureus* were grown in Mueller Hinton Broth, *S. mutans* was grown in Tryptone soya broth and *C. albicans* was grown in Sabouraud Dextrose Broth. Suspension culture of microorganism matching 0.5 McFarland



[Table/Fig-4]: The zone of inhibition by 1.25% NaOCl and 1% CHX against *S. aureus*., A: Control 1.25% NaOCl; B: Irradiated 1.25% NaOCl; C:Control 1% CHX; D: Irradiated 1% CHX

Irrigants	Groups	Zone of inhibition in mm	
		At day 1	Day 180
1.25% NaOCl	Control	13.75±0.95 *	11.75±0.28 *
	1 kGy irradiated	16.00±0.00 *	11.50±0.57 *
1% CHX	Control	14.50±0.57 * ‡	14.00±0.00 *
	1 kGy irradiated	22.00±0.00 *	15.50±0.57 * ‡

[Table/Fig-5]: Stability in antimicrobial activity of NaOCl and CHX irradiated with 1kGy against *E. faecalis*, *Values are expressed in mean±SD, * indicates significant (P<0.05) difference between the column of day 1 and day 180 for zone of inhibition. ‡ indicates significant (P<0.05) difference between day 1 control and day 180 irradiated group of 1% CHX*

Irrigants	Groups	Zone of inhibition in mm	
		At day 1	At day 180
1.25% NaOCl	Control	13.00±0.81 * #	10.25±0.5 *
	1 kGy irradiated	25.00±0.0 *	15.00±0.51 * #
1 % CHX	Control	19.00±0.0 *	16.75±0.5 *
	1 kGy irradiated	26.00±0.00 *	20.75±0.50 *

[Table/Fig-6]: Stability in antimicrobial activity of NaOCl and CHX irradiated with 1kGy against *S. aureus*, *Values are expressed in mean±SD, * indicates significant (P<0.05) difference between the columns of day 1 and day 180 for zone of inhibition., # indicates significant (P<0.05) difference between day 1 control and day 180 irradiated groups of 1.25% NaOCl and 1%CHX, respectively*

standards was used to get uniform lawn of microorganisms in the respective agar media. The *E. faecalis*, *S. aureus* and *S. mutans* were grown in Mueller Hinton Agar and *C. albicans* was grown in Sabouraud Dextrose Agar. Using sterile swabs, culture was uniformly spread over the solidified agar media. Using a sterile microtip, 20 µL of irrigants was incorporated aseptically to sterile paper disc (6mm, Hi-Media) and placed over the solidified media. Then plates were incubated for overnight at 37°C. The antimicrobial activity of irrigants was recorded and compared by measuring the zone of inhibition.

Cytotoxicity assay: Modified method of MTT {3-(4, 5 Dimethy thiazol-yl}-2, 5- Diphenyl-tetrazolium bromide) assay [18] was employed to study the cytotoxic effect of irrigants. One hundred microliters of media containing 10,000 cells were seeded into each well of 96 well microtiter plates. Then cells were allowed to grow under 5% CO_2 condition at 37°C in humidified incubator. After 24 h of incubation the media was removed and the cells were treated with 100 µl of irrigants for 10 min. After removal of irrigants, the

Groups	Irrigants	Day 1	Day 180
Control	1.25% NaOCl	0.087±0.01	0.077±0.06
	1% CHX	0.025±0.01	0.016±0.02
Irradiated 1kGy	1.25% NaOCl	0.012±0.007	0.005±0.002
	1% CHX	0.025±0.006	0.013±0.01

[Table/Fig-7]: Optical density readings of MTT assay showing cytotoxicity of irrigants, *Values are expressed in mean±SD*

cells were washed in phosphate buffered Saline (PBS, pH 7). The cytotoxicity of irrigants was evaluated by incubating the cells with 100 µL of MTT dye (0.5 mg/ml) in PBS for 4 h at 37°C in 5% CO_2 incubator. The intensity of the colour was measured by adding Dimethyl Sulphoxide (DMSO) at 545 nm using Lisa chem plate reader.

The antimicrobial and Cytotoxic activity data of irrigants before and after exposure to e-beam irradiation was analysed by one way ANOVA and Post-Hoc tests. The significance of the results were considered at 95% confidence interval ($p<0.05$). The stability of irrigants after 180 days was analysed by paired t-test and stability of irradiated irrigant at 180 day was compared with control at day 1 by independent t-test.

RESULTS

1. Physicochemical properties

The changes in physiochemical parameters, pH [Table/Fig-1], colour, sedimentation and precipitation of irrigants were not noticed on day 1 after irradiation and during the storage period of 180 d.

2. Antimicrobial activity of irrigants on Day 1

The results of this study indicate the significant increase ($p <0.001$) in the antimicrobial property of irrigants against *E. faecalis* and *S. aureus* following the e-beam irradiation [Table/Fig-2]. Among the tested concentrations, the 1.25% NaOCl and 1% CHX found to be effective concentrations to inhibit *E. faecalis* and *S. aureus* following 1 kGy irradiation [Table/Fig-3,4]. However, the significant differences were not observed in the antimicrobial activity of irrigants irradiated with higher dosages of e-beam.

3. Antimicrobial activity of irrigants after 180 days

This results showed the significant decrease ($p<0.05$) in antimicrobial activity of controls and irradiated irrigants against *E. faecalis* and *S. aureus*, except for 1% CHX against *E. faecalis* [Table/Fig-5]. However, 1% CHX irradiated with 1 kGy showed the significantly ($p=0.050$) increased antimicrobial activity against *E. faecalis* (15.50±0.57) following the storage for 180 days at 4°C compared to control on day 1 (14.50±0.57). Similar results were also observed against *S. aureus* (15.00±0.51) by using irradiated 1.25% NaOCl at 1 kGy that showed significantly ($p<0.05$) increased antimicrobial activity than their respective control (13±0.81) on day 1 [Table/Fig-6].

4. Cytotoxicity of irrigants.

The tested irrigants were found to be cytotoxic to human gingival fibroblast cells. The results showing the reduction in viable cell count is given in [Table/Fig-7]. There was no significant change in the cytotoxic effect of CHX during storage at 4°C for 180 d. In contrary, there was a significant increase in the cytotoxicity of NaOCl irradiated with 1 kGy following the storage.

DISCUSSION

In this study, antimicrobial effect of the irradiated dental irrigants NaOCl, and CHX was evaluated against common oral pathogens, *E. faecalis*, *S. aureus*, *S. mutans* and *C. albicans*. The stability in antimicrobial property of irradiated irrigants over non-irradiated irrigants was evaluated after 180 d of storage. To our knowledge, this is a first preliminary study to evaluate the effect of e-beam irradiation on antimicrobial activity of dental irrigants.

The main concern of using e-beam radiation is the degradation of the native compound leading to formation of new radiolytic intermediate or free radicals, which manifested by change in color of the irradiated substance [19]. The unchanged color of irrigants and nonappearance of sediments or precipitation following the exposure to e-beam radiation on day1 and after 180 d storage at 4°C illustrates the absence of radiolytic compounds in irradiated dental irrigants. The findings of the present study are in accordance with the published report which illustrated no change in the pH of irradiated sulphonamide [20]. However, the earlier study demonstrated the formation of free radicals by analogs of anthracycline followed by e-beam irradiation at 25 kGy [21]. Further, the complex-free radicals formed following the gamma irradiation of penicillin derived antibiotics at 25 kGy were decreased with storage time of 80 d [22].

We found that e-beam irradiation increased the antimicrobial activity of 1.25% NaOCl and 1% CHX on E. faecalis and of 1.25% NaOCl, 2.5% NaOCl, 1% CHX and 2% CHX on *S. aureus*. In contrary, the previous studies conducted on antibiotics did not show any significant changes in their biological property after exposure to gamma irradiation [1,19]. This difference might be either due to the state of the substance used for radiation or the source of radiation.

The e-beam irradiation is used to break down the detergent based pollutants to reduce their adverse biological effects for example; the acute toxicity of sodium dodecyl sulfate was significantly decreased after e-beam irradiation at 3 and 6 kGy [3]. Further, it has also been reported that the cytotoxicity of pharmaceutical compound diclofenac decreased at higher doses of e-beam irradiation against Vibrio fisheri [23]. In contrast, our study showed no changes in the cytotoxicity of irradiated CHX, but the cytotoxic effect of irradiated NaOCl was significantly increased ($p<0.001$). Further, there were no changes in the cytotoxicity of irradiated and non-irradiated irrigants even after 180 d of storage. Though, the antimicrobial property of both control and irradiated irrigants decreased after 180 d, the irradiated irrigants were comparatively more stable than the respective control groups.

CONCLUSION

With this preliminary study it can be concluded that e-beam irradiation enhances the antimicrobial property of dental irrigants, NaOCl and CHX at 1 kGy. In addition, no changes in the cytotoxicity of irradiated dental irrigants were observed. The increased antimicrobial activity of irradiated irrigants should be further confirmed by evaluating against wide range of microorganisms, including gram negative bacteria, anaerobic bacteria and fungal species. Moreover, the formation of radiolytic substances or free radicals was assessed through the changes in pH, color, and visualisation of precipitation or sedimentation. Hence, the attempts are presently being made to further analyse and confirm the above observations by chromatographic methods.

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