Effect of electron-beam irradiation on antimicrobial, antibiofilm activity, and cytotoxicity of mouth rinses

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ABSTRACT

Background: Oral health diseases are common in all regions of the world. Mouth rinses are widely used generally by population as a part of daily oral care regimen. In addition to antimicrobial activity, mouth rinses possess certain cytotoxic effects. Electron-beam (E-beam) radiation is a form of ionizing energy known to induce structural, physical, and chemical changes in irradiated products. In this study, the modulatory effects of E-beam in irradiated mouth rinses were evaluated for its biological activities.

Materials and Methods: The antimicrobial activities of nonirradiated and irradiated mouth rinses were evaluated for its antimicrobial and antibiofilm activities against oral pathogens, Enterococcus faecalis, Streptococcus mutans, Staphylococcus aureus, and Candida albicans. The antimicrobial activity was evaluated by disc diffusion method and antibiofilm activity was evaluated by O’Toole method. The cytotoxicity was evaluated against human gingival fibroblast (HGF) cells by 3-(4, 5 Dimethythiazol-yl)-2,5-Diphenyl-tetrazolium bromide assay.

Results: Colgate Plax (CP) exhibited the antimicrobial activity against the tested pathogens, and a significant ($P < 0.05$) increase was observed against $S. aureus$ at 750 Gy irradiation. Further, CP significantly ($P < 0.05$) suppressed $S. mutans$, $S. aureus$, and $C. albicans$ biofilm. Listerine (LS) inhibited $S. mutans$ and $C. albicans$ biofilm. Whereas irradiated CP and LS significantly ($P < 0.05$) suppressed the biofilm formed by oral pathogens. The suppression of biofilm by irradiated mouth rinses was dose- and species-dependent. There was no significant ($P > 0.05$) difference in the cytotoxicity of irradiated and nonirradiated mouth rinses on HGF cells. However, an increased percentage viability of HGF cells was observed by mouth rinses irradiated at 750 Gy.

Conclusion: The E-beam irradiation enhanced the antibiofilm activity of mouth rinses without modifying the cytotoxicity.

Key words: Antibiofilm activity, antimicrobial activity, cytotoxicity, electron-beam irradiation, mouth rinses

Oral health diseases are common in all regions of the world. Oral diseases such as dental caries, periodontal diseases, and tooth loss are among the major public health problems.[1] Dental caries are infectious diseases caused by the dynamic and extremely complex oral biofilm on tooth which is called dental plaque. It results in demineralization of hard tissue of tooth as a response to the microbial challenge.[2] Periodontal diseases are initiated by the accumulation of microbial plaque above the gingival margin, which extends into the sub-gingival environment. It induces an inflammatory response in the tissue, redness, swelling, and pain.[3]

Mouth rinses are often used by the general population in conjunction with brushing and flossing as a part of daily oral care regimen to prevent or minimize microbial accumulation.[4] Electron-beam (E-beam) radiation, a
form of ionizing energy, has been introduced as a means of sterilizing single-use, disposable health-care products. Ionizing radiation induces structural changes in the native pharmaceutical compound resulting in altered physico-chemical, microbiological, and toxicological properties. It has also been reported to be an effective tool to decompose the organic substances and reduce the toxicity.

In this present study, modulatory effect of E-beam on antimicrobial and cytotoxicity of mouth rinses was evaluated on selected oral persistent pathogens Enterococcus faecalis, Staphylococcus aureus, Streptococcus mutans, and Candida albicans, and human gingival fibroblast (HGF) cell line.

MATERIALS AND METHODS

Micro-organisms and maintenance
The type strains of E. faecalis (ATCC 29212), S. aureus (ATCC 29213), S. mutans (MTCC 890), and C. albicans were obtained from Nitte University Centre for Science Education and Research, Nitte University, India. Bacterial culture media Mueller Hinton Broth (MHB), Sabouraud Dextrose Broth (SDB), Tryptone Soya Broth (TSB), Mueller Hinton Agar (MHA), and Sabouraud Dextrose Agar (SDA), were procured from HiMedia, Mumbai, India.

Cell line and maintenance
HGF cell line was obtained through explants culture of healthy gingival tissue. The cell line was sub-cultured 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 amphotericin B at 37°C and 5% CO₂ in humidified incubator. Cell culture medium DMEM, fetal bovine serum, antibiotics, and chemicals were procured from HiMedia. Petri plates, tissue culture flasks, and microtiter plates were obtained from Tarsons, Mumbai, India.

Mouth rinses
Commercially available Colgate Plax (CP) and Listerine (LS) were used without modifications in this study.

Electron-beam irradiation of irrigants
The irrigants were subjected to E-beam radiation at Microtron Centre, Mangalore University, Karnataka, India. The mouth rinses (10 ml) packed in sterile polythene pouches were exposed to 250 Gy, 500 Gy, 750 Gy, and 1000 Gy of E-beam irradiation at a dose rate of 500 Gy/min. The irradiated and nonirradiated mouth rinses were evaluated for their physico-chemical, antimicrobial, and cytotoxic effects.

Antimicrobial activity by disc diffusion method
In disc diffusion method, the suspension culture of E. faecalis and S. aureus was grown in MHB, S. mutans was grown in TSB, and C. albicans was grown in SDB. Suspension culture of micro-organism matching 0.5 McFarland standards was used to get uniform lawn of micro-organisms in the respective agar media. The E. faecalis, S. aureus, and S. mutans were grown in MHA, and C. albicans was grown in SDA. Bacterial and fungal culture was uniformly spread over the solidified agar media using sterile swabs. About 20 µL of mouth rinses were incorporated aseptically to sterile paper disc (6 mm, HiMedia) and placed over the solidified media. Then, culture plates were incubated for overnight at 37°C. The antimicrobial activity of mouth rinses was recorded by measuring the zone of inhibition (ZOI).

Antibiofilm assay
Biofilm was grown in microtiter plate as described previously by O’Toole. The wells of the microtiter plates were seeded with 100 µl of 18 h old bacterial cultures diluted at 1:100 ratios in respective broths and incubated at 37°C for 24 h. Then, the excess broth was discarded carefully using a multichannel pipettor and washed in 3 times with phosphate buffer saline (PBS, pH 7) and air dried. Following the air drying, 20 µL of irradiated and nonirradiated mouth rinses were added to each wells containing biofilm and incubated for 10 min at 37°C. Then, the test materials were discarded, washed with distilled water for 3 times, and air dried at room temperature. The biofilm was stained by incubating with 125 µL of 0.1% crystal violet stain for 10–15 min at room temperature. Stain was removed by three washes in doubled distilled water and air dried. Destaining of the biofilm was done by incubating with 30% acetic acid for 15 min at room temperature. Quantification of the biofilm was performed at 600 nm using Lisa chem plate reader.

Cytotoxicity
3-(4, 5 Dimethylthiazol-yl)-2,5-Diphenyl-tetrazolium bromide (MTT) assay was employed to study the cytotoxic effect of mouth rinses. About 100 µL of media containing 10,000 cells were seeded to each well of 96-well microtiter plates. Then, the cells were allowed to grow under 5% CO₂ at 37°C in humidified incubator. After 24 h of incubation, the media was removed and the cells were treated with 20 µL of mouth rinses for 10 min. After the removal of mouth rinses, the cells were washed in PBS (pH 7). The cytotoxicity of mouth rinses was evaluated by incubating the cells with 100 µL of MTT dye (0.05 mg/ml) in PBS for 4 h at 37°C in 5% CO₂ incubator. The intensity of the color was measured by adding dimethyl sulphoxide at 545 nm using Lisa chem plate reader.

Physico-chemical parameters
The color, sedimentation, precipitation of control, and irradiated mouth rinses were recorded immediately after E-beam irradiation. The pH of mouth rinses was recorded using pH strips (Fisher Scientific) of range 2–10.
**Statistical analysis**

The antimicrobial activity and cytotoxicity data were statistically analyzed by one-way ANOVA and post hoc tests using GraphPad Prism (version 3.02, GraphPad Software Inc., San Diego). The significance of the results was considered when \( P < 0.05 \), \( < 0.01 \), and \( < 0.001 \).

**RESULTS**

**Antimicrobial activity of Colgate Plax against oral pathogens before and after irradiation**

The data describing the antimicrobial activity of CP against oral pathogens are presented in Table 1 as mean ± standard deviation (SD). CP was found to be more active against \( S. \) mutans and moderately active against other pathogens. A significant \( (P < 0.05) \) increase in the antibacterial activity of irradiated CP was observed at 750 Gy against \( S. \) aureus. However, E-beam irradiation of CP did not cause any significant \( (P > 0.05) \) difference in antimicrobial activity against \( E. \) faecalis and \( C. \) albicans after radiation. Although there was a significant \( (P < 0.001) \) decrease at lower doses, the antimicrobial activity against \( S. \) mutans was found to be insignificant \( (P > 0.05) \) at 1000 Gy compared to nonirradiated group.

**Antibiofilm activity of nonirradiated and irradiated mouth rinses**

The antibiofilm activity of nonirradiated and irradiated CP and LS is presented in Table 2 and 3, respectively. In nonirradiated groups, the suppression of \( C. \) albicans biofilm by CP and \( S. \) mutans and \( C. \) albicans biofilms by LS was found to be highly significant \( (P < 0.001) \). However, no significant \( (P > 0.05) \) differences were observed against \( E. \) faecalis biofilm by both CP and LS. Further, the suppression was significant \( (P < 0.05 \) and \( P < 0.01 \)) against \( S. \) aureus and \( S. \) mutans biofilms by CP.

E-beam irradiated CP and LS were highly effective in reducing the activity of \( E. \) faecalis biofilm at all doses, and the observed differences were statistically significant \( (P < 0.001) \). \( S. \) aureus biofilm was significantly \( (P < 0.01) \) suppressed by irradiated LS at 250 Gy and 500 Gy, whereas in CP, significant differences were observed at 500 Gy onward. The irradiated CP significantly \( (P < 0.001) \) suppressed the \( S. \) mutans and \( C. \) albicans biofilm at all doses. The LS irradiated at 250–750 Gy resulted in a highly significant \( (P < 0.001) \) suppression of \( C. \) albicans biofilm. Moreover, both irradiated CP and LS were highly effective in suppressing the \( E. \) faecalis biofilm compared to nonirradiated counterparts, and the differences were found statistically significant \( (P < 0.001) \).

Viability of human gingival fibroblasts in irradiated and nonirradiated mouth rinses

The viability results of HGF by irradiated and nonirradiated CP and LS are presented in Figures 1 and 2. Overall, a decrease in mean ± SD of HGF was observed upon treating

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**Table 1: Zone of inhibition by Colgate Plax before and after irradiation at different doses**

| Pathogens              | Control  | 250 Gy  | 500 Gy  | 750 Gy  | 1000 Gy |
|------------------------|----------|---------|---------|---------|---------|
| *Enterococcus faecalis*| 15.75±0.5| 14.75±0.5| 14.50±0.5| 15.50±0.5| 14.50±0.5|
| *Staphylococcus aureus*| 14.00±0.81| 16.00±0.0| 16.00±0.0| 16.00±0.57*| 16.00±0.81|
| *Streptococcus mutans* | 22.00±0.0| 18.50±0.57| 18.50±0.57| 18.50±0.57| 21.75±0.5 |
| *Candida albicans*     | 14.75±0.5| 13.75±0.5| 13.75±0.5| 13.50±0.57| 14.25±0.5 |

*Significant \( (P<0.05) \) increase in zone of inhibition

**Table 2: Suppression of biofilm by nonirradiated and irradiated Colgate Plax**

| Pathogens              | Control | Nonirradiated CP | Irradiated groups  |
|------------------------|---------|------------------|--------------------|
|                        |         |                  | 250 Gy  | 500 Gy  | 750 Gy  | 1000 Gy |
| *Enterococcus faecalis*| 0.423±0.005| 0.391±0.01| 0.339±0.02*| 0.322±0.017*| 0.324±0.019*| 0.324±0.013*|
| *Staphylococcus aureus*| 0.537±0.012| 0.443±0.03*| 0.445±0.07| 0.425±0.04*| 0.416±0.03*| 0.416±0.01*|
| *Streptococcus mutans* | 0.440±0.019| 0.369±0.03*| 0.341±0.03*| 0.337±0.02*| 0.324±0.02*| 0.335±0.007*|
| *Candida albicans*     | 0.450±0.006| 0.307±0.03*| 0.330±0.02*| 0.325±0.02*| 0.308±0.01*| 0.324±0.011*|

*Significant difference between control and respective irradiated groups at \( P<0.001 \). †Significant difference between control and respective nonirradiated group at \( P<0.05 \). ‡Significant difference between control and respective irradiated groups at \( P<0.01 \). §Significant difference between control and respective nonirradiated group at \( P<0.001 \). ††Significant difference between control and respective irradiated groups at \( P<0.001 \). †‡Significant difference between control and respective nonirradiated group at \( P<0.01 \). †§Significant difference between control and respective irradiated groups at \( P<0.001 \). CP=Colgate Plax

**Table 3: Suppression of biofilm by nonirradiated and irradiated listerine**

| Pathogens              | Control | Nonirradiated LS | Irradiated groups |
|------------------------|---------|------------------|------------------|
|                        |         |                  | 250 Gy  | 500 Gy  | 750 Gy  | 1000 Gy |
| *Enterococcus faecalis*| 0.423±0.005| 0.422±0.01| 0.346±0.02*| 0.350±0.006*| 0.342±0.007*| 0.356±0.007*|
| *Staphylococcus aureus*| 0.537±0.012| 0.456±0.002| 0.420±0.02*| 0.431±0.035*| 0.462±0.04*| 0.486±0.04*|
| *Streptococcus mutans* | 0.440±0.019| 0.341±0.01*| 0.340±0.01| 0.378±0.03*| 0.375±0.02*| 0.378±0.01*|
| *Candida albicans*     | 0.450±0.006| 0.328±0.01*| 0.358±0.02*| 0.374±0.01*| 0.381±0.01*| 0.398±0.02*|

*Significant difference between control and respective irradiated groups at \( P<0.001 \). †Significant difference between control and respective irradiated groups at \( P<0.01 \). ‡Significant difference between control and respective nonirradiated groups at \( P<0.001 \). §Significant difference between control and respective irradiated groups at \( P<0.001 \). ††Significant difference between control and respective nonirradiated group at \( P<0.001 \). †‡Significant difference between control and respective irradiated groups at \( P<0.001 \). †§Significant difference between control and respective nonirradiated group at \( P<0.001 \). †‡†Significant difference between control and respective irradiated groups at \( P<0.001 \). LS=Listerine
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with irradiated and nonirradiated CP and LS. Both mouth rinses were highly effective in significantly ($P < 0.001$) reducing the number of viable HGF. Particularly, both irradiated CP and LS were found to be more effective in their activity against HGF, and the differences were highly significant ($P < 0.001$). Although there was no significant ($P > 0.05$) difference in nonirradiated and irradiated mouth rinses, the data showed a slight increase in the viability of cells treated with mouth rinses irradiated at 750 Gy.

Discoloration of mouth rinses after electron-beam irradiation

The gradual discoloration of mouth rinses CP and LS upon E-beam irradiation is shown in Figure 3a and b, respectively.

DISCUSSION

It is well known that the oral diseases are a worldwide health concern with a considerable impact on public. Further, due to an increasing intake of sugars in the diet, use of tobacco, inadequate exposure to fluorides, and lack of access to dental care, it is expected that the incidence of dental caries and periodontal disease will continue to increase. S. mutans is a primary etiological agent of dental caries. E. faecalis is an opportunistic pathogen, S. aureus and C. albicans are also isolated from persistent apical periodontal lesions. Oral microflora has been considered as a critical factor in both caries and periodontal disease, and cause the disease pathogenesis mainly by producing the biofilms. Hence, the use of different types of mouth rinses has been given a due importance to act against the harmful micro-organisms associated with oral diseases or infections. However, concerns regarding the development of antibiotic-resistant strains and adverse effects of contemporary mouth rinses have led to the interests in the use of nonconventional or alternative medicines and plant extracts. A few reports have also suggested the possible use of plant extracts in oral care for effective and efficient inhibition of microflora by natural antimicrobials. However, the potential benefits of chemotherapeutic formulations in mouth rinses provide impetus for research in finding effective mouth rinses for oral care.

The present study evaluated the modulatory effect of E-beam on antimicrobial effect of mouth rinses, CP and LS on oral persistent pathogens E. faecalis, S. aureus, S. mutans, and C. albicans. Further, the cytotoxic effect of two mouth rinses was also determined on the cultured HGF. The results showed that CP was more effective against all tested pathogens by producing a clear ZOI. This may be due to the presence of active component cetylpyridinium chloride, which is a quaternary, nonionic surfactant with broad-spectrum antimicrobial activity. Whereas the mouth rinse LS did not show a ZOI against the tested micro-organisms. The inefficacy of LS against oral pathogens in our study is in concurrence with the earlier report by Aneja et al., suggesting that different formulations of constituents in mouth rinses might be responsible for varied effects.

Biofilm experiments were performed to evaluate the effect of antimicrobial components in selected mouth rinses. The results showed a greater antibiofilm activity of LS compared to CP against the tested oral pathogens. These data are in consistence with published report where the efficacy of
mouth rinses containing essential oils was observed to be more than cetlypyridinium chloride formulations.\(^{18,19}\)

E-beam is a form of ionizing radiation used in food and pharmaceutical industries as a mean of sterilization. Several studies have reported the chemical stability of pharmaceutical drugs or antibiotics after irradiation.\(^{20,21}\) However, there are no reports on the effects of ionizing radiation on biological activities (antimicrobial and cytotoxicity) of pharmaceuticals. Therefore, in this study, value addition of E-beam radiation on biological properties of mouth rinses was evaluated.

The mouth rinses were exposed to lower doses of E-beam irradiation because higher doses might degrade the active component.\(^{22}\) A significant increase in the antimicrobial activity of CP was observed against S. aureus upon irradiation at 750 Gy. Irradiation induced the higher efficacy of biofilm suppression by CP and LS. Irradiated CP and LS suppressed E. faecalis biofilm more effectively than the nonirradiated counterparts. Further, irradiation increased the efficacy of biofilm suppression by mouth rinses.

Any antimicrobial agents are expected to have minimal cytotoxic effect on host cells. In this study, the results demonstrated the cytotoxic effect of both CP and LS on cultured HGF. These results are in accordance with the earlier reports on CP and LS.\(^{23,24}\) However, no significant differences were observed in the reduction of HGF cells by irradiated and nonirradiated mouth rinses. Importantly, an earlier report on the effect of E-beam on sodium dodecyl sulfate demonstrated the reduction in toxicity of the surfactant.\(^{6}\)

During technological processing, the majority of drugs and therapeutic substances can be negatively affected by radiation. This can be manifested by change in color or precipitation or sedimentation of the irradiated substance.\(^{20}\) A gradual discoloration in mouth rinses irradiated from 250 Gy to 750 Gy and a complete discoloration at 1000 Gy of irradiation indicated the changes in chemical properties of mouth rinses upon E-beam radiation [Figure 3]. However, there were no changes in pH, as it was observed to be 6 for CP and 4 for LS before and after irradiation. These results indicate that radiation did not cause the formation of acidic or basic intermediates. These observations are in contrary with the reported work on sulfonamides,\(^{5}\) where color stability and change in pH were observed.

**CONCLUSION**

The present study demonstrated that the mouth rinse CP had the highest antimicrobial property against the selected oral pathogens, whereas LS was effective in suppressing the tested biofilms. E-beam component did not alter the antimicrobial properties of the mouth rinses much, but it enhanced the biofilm activity without modifying the cytotoxic effects on fibroblast cells. Thus, E-beam irradiation can be a useful processing tool to enhance the antimicrobial activities of contemporary mouth rinses. Further studies may be directed to investigate the cytotoxicity of these mouth rinses under *in vivo* conditions.

**Acknowledgment**

The authors gratefully acknowledge the financial support extended by the Department of Atomic Energy, Board of Research in Nuclear Sciences.

**Financial support and sponsorship**

The BRNS-RTAC grant (Sanction No: 2010/35/BRNS with RTAC) of the Department of Atomic Energy, Board of Research in Nuclear Sciences, provided the financial support for this work.

**Conflicts of interest**

There are no conflicts of interest.

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