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Abstract

Aim: The aim of this study was to provide preliminary data on the most effective erythrosine concentration and light dose for the erythrosine-based photodynamic therapy (PDT) of oral plaque biofilms formed *in vivo*.

Method: A randomized controlled study with 15 volunteers was carried out to investigate the effect of photosensitiser and light dose on the killing of bacteria in oral plaque biofilms formed *in vivo*. All volunteers wore a removable *in situ* appliance carrying 6 enamel slabs for two phases of two weeks each. During this time, plaque biofilms accumulated on the enamel slabs. The slabs were then removed from the appliances for PDT-treatment *in vitro*. In the first phase of the study, erythrosine doses of 22 and 220uM were used for the photodynamic treatment of the biofilms. In the second phase, the erythrosine concentration was kept constant, and the light dose was varied. Following treatment, the biofilms were disaggregated, and the total bacterial killing determined using colony counting.

Results: The erythrosine dose of 220uM caused the most cell killing relative to controls. Fifteen minutes continuous irradiation with light and light fractionation of 5x1 minute irradiation separated by 2 minute dark recovery periods were found to be the most effective bactericidal regimes.

Conclusion: Erythrosine-based PDT shows promise as an anti-bacterial treatment for oral plaque biofilms. Further research is needed to prove its clinical and cost-effectiveness compared with current best practice.

Introduction

According to Marsh (2004) dental plaque can be defined as “the diverse community of microorganisms found on the tooth surface as a biofilm, embedded in an extracellular matrix of polymers of host microbial origin”. Dental plaque is a biofilm, consisting of a diversity of co-aggregated bacteria (Al Ahmad et al., 2007; Dige et al., 2007). The commensal bacteria of dental plaque microflora (early colonisation) are important for the preservation of oral health and avoidance of oral disease (Socransky et al, 2002; Kumar et al, 2005). Ecological shifts of commensal dental microflora (colonisation of early colonisers by late colonisers) due to environmental perturbations, such as excessive sugar intake, plaque over accumulation, can lead from dental health to caries and periodontal diseases (Allaker and Douglas, 2009).

Toothbrushing and use of topical antibacterials are the conventional methods for dental plaque removal and control. However, these methods are not always effective, appropriate or possible. The majority of children, adolescents and adults do not accomplish a standard of daily plaque control consistent with good oral health. In addition, mechanical plaque removal via toothbrushing may not be tolerated by specific patient groups such as those with physical disabilities, the medically compromised (such as patients with Epidermolysis bullosa) and younger children. Increasingly, there is also concern about the limited access of topical antibacterial agents to plaque biofilms and bacterial resistance to both antibacterials and antibiotics (Sreenivasan and Gaffar, 2002).

Therefore, it would be advantageous to develop alternative strategies for the prevention of dental caries and periodontal disease. One such alternative is photodynamic therapy (PDT). PDT involves the use of a photoactive dye (photosensitiser) that binds to, or it is taken up by the target cells and can be activated by light of a suitable wavelength. The transfer of energy from the activated photosensitiser to available oxygen results in the formation of toxic oxygen species, which can damage target tissues (Meisel and Kocher, 2005). At the doses used, either light or photosensitiser alone are used to facilitate targeting of the treatment by the appropriate application of light.

Erythrosine is one of the photosensitisers that have been used in PDT with a view to control dental plaque biofilms. Erythrosine is already used successfully in the dental clinic as a plaque disclosing solution. Therefore, erythrosine-based PDT may be an adjunct to the present methods of management of dental plaque biofilms or may be a new option in cases where the existing management approaches for dental plaque biofilms cannot be carried out.

Wood *et al.*, (2006) in an *in vitro* study assessed the light activated bacterial killing efficacy of erythrosine with that of two other photosensitisers (photofrin and methylene blue). *Streptococcus mutans* biofilms with a thickness of 200 µm were incubated with each photosensitiser having a 22 µM concentration. The biofilms were then irradiated with white light for 15 minutes. Erythrosine was found to be more effective than both photofrin and methylene blue.

Metcalf *et al.*, (2006) who also used erythrosine-based PDT demonstrated that fractionation of the same overall light dose applied to *Streptococcus mutans* biofilms *in vitro*, increased the amount of bacterial killing compared with that caused by the same overall continuous light dose. Further fractionation of the light dose resulted in a further rise in bacterial killing compared with the continuous irradiation protocol.

According to Goulart *et al* (2010) erythrosine was more effective at killing bacterial cells of *Aggregatibacter actinomycetemcomitans* in planktonic and biofilm cultures compared with methylene blue, when irradiated with an odontologic resin photopolymeriser *in vitro*.

While the use of erythrosine in the management of isolated, specific oral plaque bacteria, growing in planktonic and/or biofilm cultures *in vitro* seems to be promising, it is important to study the effect of erythrosine on natural undisturbed oral plaque biofilms that have been formed *in vivo*, as this is closer to the clinical situation.

Therefore, the focus of the present study was to determine *in vitro* a) the most effective bactericidal concentration of erythrosine-based PDT on dental plaque biofilms formed *in vivo* and b) the most effective irradiation time for the PDT treatment of the biofilms.

Materials and Methods

This study was conducted in two phases (Fig 1). Phase I of the study involved determining the most effective bactericidal concentration of erythrosine-based PDT on dental plaque biofilms. Phase II of the study consisted of studying the most effective irradiation time for the PDT treatment of biofilms.

Participants

Ethical approval was obtained by the South Yorkshire Research Ethics Committee (10/H1310/23). . Fifteen healthy volunteers aged ≥ 18 years old with presence of at least 20 natural teeth, free from visual signs of untreated caries and periodontal disease or any other adverse dental/oral health conditions, , salivary flow rate ≥ 0.25 ml/min and with no relevant medical history were recruited in the study. All volunteers received verbal and written information concerning the study and gave written consent to participate. A dental scale and polish was given to the volunteers prior to taking impressions to construct a lower removable appliance. Participants were instructed to wear their appliances at all times except when eating, drinking and toothbrushing for the duration of the study. At these times volunteers had to place the appliances in a damp gauze inside a plastic case to prevent the accumulated plaque from drying out.

Sample size determination

The study aimed to determine the most effective erythrosine concentration and irradiation time for erythrosine-based PDT by comparing the percentage reductions of the total bacterial counts in human plaque samples among the control and treatment groups. As there were no similar studies in the literature, the sample size determination was based on previous estimates of *Streptococcus mutans* (Cury et al., 2001), which constitutes a basic microorganism of human dental plaque. Adjusting for multiple comparisons by lowering the significance level at $\alpha = 0.005$ with a power of 0.8 the power calculation resulted in a sample size of at least 15 per group.

Teeth selection and enamel slabs preparation

Sound upper and lower premolars were obtained from the Leeds Dental Institute's Tissue Bank. The premolars were mounted in yellow stick impression compound

(Kerr, UK) on cutting discs. Each premolar was sectioned by a Well Precision water cooled Diamond Wire Saw (Walter Ebner, Le Locle, Germany) and the buccal and lingual surfaces of each premolar were separated. Three enamel slabs of about 3 mm width, 2 mm length and 2 mm depth were produced from the buccal section and another three slabs of exactly the same dimensions were produced from the lingual section of each tooth. All six slabs produced from the same premolar were used in the same appliance. Once the enamel slabs were prepared, they were kept moist in distilled water with thymol in micro-centrifuge tubes sealed with sealing film (Parafilm M, SPI, USA). They were then sent to the Department of Immunology of the University of Liverpool, where they were exposed to gamma irradiation (4080 Gy) for sterilisation. This level of exposure provides sterilization without altering the structural integrity of enamel slabs.

Preparation of in situ appliances

A mandibular removable appliance (Fig 2) was used that consisted of a labial arch wire, acrylic flanges buccally on premolars and first permanent molars and a U clasp to each of the first permanent molars. Three enamel slabs were inserted in the right flange and another three in the left flange of each appliance. It is important to note that a 4 mm deep space was made for each enamel slab to be placed in the appliance, leaving a 1 mm depth for plaque accumulation on the surface of the slab. Also a superficial well of 2 mm diameter was made at the lingual surface of the flange to accommodate slab removal. Slabs were secured in position with sticky wax (Bredent, Germany) and care was taken not to cover the exposed surface of the enamel. The distance between every two slabs in the same flange varied from 3 to 7 mm. Block randomization based on a random table of numbers was used to randomly allocate every 6 slabs to the *in situ* appliance.

All six enamel slabs were removed in the laboratory at the end of each two week phase of the study and were randomly allocated to the control and treatment groups. Each one of the six slabs with its accumulated plaque was transferred to a separate well of a 12-well tissue culture plate. Each slab was stabilized with petroleum jelly and the area of the slab with the accumulated plaque was uppermost in the well.

A summary of the protocol of the present study, following removal of the enamel slabs from the appliances, can be seen in figure 1.

Phase I of the study included 15 volunteers divided into three groups: the negative control group (no erythrosine, no light), the group of 22 μM erythrosine and 15 minutes light (treatment group) and the group of 220 μM erythrosine and 15 minutes light (treatment group). The two test groups, consisting of one enamel slab each, were incubated with the designated erythrosine solutions for 15 minutes and irradiated for a period of 15 minutes. On completion of this procedure the standard microbiological techniques mentioned previously were applied in order to estimate the total bacterial counts per group.

Following completion of phase I percentage reductions of the total bacterial counts were estimated to determine the most effective bactericidal concentration of erythrosine among 0, 22 and 220 μM erythrosine. That concentration was used in phase II of the study.

The 2nd phase of the study included the same 15 subjects. For each one of the volunteers there were six groups to be compared. Each group consisted of one slab with accumulated plaque. The first two groups representing the negative (no erythrosine, no light) and positive control groups (220 μM erythrosine, no light) were covered with 2 ml of Reduced Transport Fluid and 220 μM erythrosine respectively. The remaining four groups, which were the treatment groups, were covered with 2 ml of 220 μM erythrosine each. Again each slab from each group was incubated for 15 minutes in its solution and after that time, the positive and the negative control groups were not irradiated, whereas each one of the four treatment groups had continuous irradiation either for 2 or 5 or 15 minutes or light fractionation of 5 \times 1 minute separated by dark recovery periods of 2 minutes.

PDT treatment

All slabs with plaque samples were incubated for 15 minutes in the respective solutions (2 ml of either Reduced Transport Fluid or erythrosine). The 12-well plate was covered with foil throughout the incubation period. Following the 15 minutes incubation, the foil was removed and the 12-well plate was placed underneath a 400

W tungsten filament lamp with an output of 22.7mW/cm² in the absorbance range of erythrosine (535nm). A heat-dissipating water bath was positioned between the 12-well plate and the lamp to prevent hyperthermic effects. The wells of the 12-well plate containing the control slabs and plaque that was not to be irradiated were covered with foil during this time.

On completion of the irradiation regime the 2 ml solution with the enamel slab and accumulated plaque from each well was transferred to a plastic vial and vortexed for 30 seconds with 5-6 glass beads to disrupt the biofilms. Dilution series were prepared and spread on blood agar plates.

The blood agar plates were placed in a CO₂ incubator and incubated for 48 hours. Total bacterial counts were then determined. Only plates in which the total bacterial counts ranged from 30 – 300 were used for comparisons among the treatment and control groups.

Statistical analysis

Collected data were compiled into spreadsheets and statistical analysis was carried out using the SPSS statistical package for Windows version 17 (SPSS Inc. Illinois). A significance level of $\alpha < 0.05$ was adopted. All continuous data were checked for normality. The non-parametric Kruskal-Wallis and Mann-Whitney U tests were carried out for all skewed continuous data to compare medians. In addition, adjustment for multiple comparisons was applied by lowering the level of significance (α') according to the following equation $\alpha' = \alpha/\text{number of comparisons}$.

Results

Results of phase 1

Fifteen healthy volunteers with a mean age of 41 years participated. It can be seen from table 1 that the mean and median percentage reduction of total bacterial counts for both the 22 and 220 μM erythrosine groups were not equal. This finding meant that the data from this study were not normally distributed and therefore the median percentage reduction of total bacterial counts had to be used for each group.

According to Fig 3 there was no percentage reduction of the total bacterial counts in

the control group, whereas a percentage reduction was obvious in the 22 and 220 μM erythrosine groups. The 22 μM erythrosine group had a wider variation in the percentage reduction of the total bacterial counts compared with the 220 μM erythrosine group. Overall, the results showed that photodynamic therapy caused bacterial cell reduction in an erythrosine dose-dependent manner. The Kruskal – Wallis test showed that there was a statistically significant difference in the median percentage reduction of the total bacterial counts among the control, 22 and 220 μM erythrosine groups. Mann-Whitney U test showed that that the difference in the percentage reduction of the total bacterial counts caused by the 220 μM erythrosine group was statistically significant when compared with the 22 μM erythrosine group ($p < 0.001$). Adjustment for multiple comparisons was applied with $\alpha' = 0.05/3 = 0.017$. The 220 μM erythrosine that was found to be the most effective bactericidal was used in phase 2 of the study.

Results of phase 2

The aim of phase 2 was to determine the most appropriate irradiation time for erythrosine that would significantly reduce total viable bacterial counts among control and treatment groups. Table 2 provides descriptive statistics for the percentage reduction of total bacterial counts. As can be seen from Fig 4 no percentage reduction of the total bacterial counts could be seen in the negative control group, whereas a small percentage reduction was present in the positive control group. The 2 minutes irradiation group had the widest variation and the 15 minutes the narrowest variation in the median percentage reduction of the total bacterial counts among the irradiation groups. Overall, the results showed that photodynamic therapy caused bacterial cell reduction in a light dose-dependent manner. This was shown by the observed percentage decrease of the total bacterial counts following treatment at 2, 5, 5*1 (light fractionation of 5*1 minute separated by dark recovery periods of 2 minutes) and 15 minutes irradiation when compared with the untreated samples. As the data were not normally distributed the non-parametric Kruskal Wallis test was used. A statistically significant difference was found in the median percentage reduction of the negative control, positive control, 2, 5, 5*1 and 15 minutes groups. Following adjustment for multiple comparisons the new alpha value was set at $\alpha' = 0.05/15 = 0.003$ (where 0.05 was the previous alpha (α) and 15 the number of comparisons). No statistically

significant differences of the percentage reduction of the total bacterial counts were seen between the negative and positive control groups with a p value of 0.126, the 2 and 5 minutes continuous irradiation groups with a p value of 0.01, the 5*1 and 15 minutes irradiation groups with a p value of 0.016. Interestingly, the difference noted on the percentage reduction of the total bacterial counts for the group with continuous irradiation of 15 minutes and light fractionation of 5×1 minute separated by dark recovery periods of 2 minutes was not found to be statistically significant. Therefore, both these irradiation regimes were found to be the most effective for bacterial killing when 220 µM erythrosine was used.

Discussion

The basic concept for the present study was based on the previous studies carried out by Wood et al., (2006) and Metcalf et al., (2006) using erythrosine-based PDT on *Streptococcus mutans* biofilms. Similar to the above studies the incubation time for plaque biofilms in erythrosine was 15 minutes and the light source was a tungsten filament lamp. The current study investigated the bactericidal effect of erythrosine-based PDT that was applied to dental plaque biofilms formed *in vivo* rather than *in vitro*.

The results obtained from the study showed that *in vitro* erythrosine-based PDT reduced the total bacterial counts on *in vivo* dental plaque biofilms in an erythrosine dose-dependent and light dose-dependent manner. This was expected as the higher the concentration of the photosensitiser (Konopka and Goslinski, 2007) and the larger the duration of the light (Metcalf et al., 2006) that is used, the more the damage to the target tissues is expected.

While an irradiation time of 15 minutes would not be realistic in a clinical setting, the bacterial killing observed following the 5*1 and the 15 minutes continuous light groups was found to be not statistically significant different and therefore both these irradiation regimes were found to be equally effective. The possible explanation for this finding might be the fact that the dark periods allow time for the biofilm to

become re-oxygenated and/or that there is a redistribution or replenishment of the erythrosine within the biofilm during this time meaning that the effectiveness of the treatment is enhanced when the irradiation is resumed (Metcalf et al., 2006).

The above finding is of clinical significance as it could mean that an irradiation time of a total of 5 minutes, fractionated in 5 sets of one minute irradiation where each 1 minute set is separated by 2 minutes dark periods, might lead to an efficacious antibacterial outcome similar to that of the 15 minutes continuous light irradiation from the same light source *in vivo*.

It was also shown that 220 μM erythrosine was the most effective bactericidal concentration leading to a 98% reduction of the total bacterial counts when compared with untreated samples (no reduction) and samples treated with 22 μM erythrosine (91% reduction). Nevertheless, it must be taken into consideration that PDT generally affects all of the tissue in which the photosensitiser is localized (Moor et al., 2003), although the severity of these effects will be dose-dependent. In addition, application of a higher concentration of photosensitiser has been reported to be more cytotoxic than that of a photosensitiser with a lower concentration (Moor et al., 2003). If the photosensitiser is cytotoxic even without activation with light, selectivity can be increased through conjugation with, for example, antibodies. This approach has shown promising results for both anticancer (Konan et al., 2002) and antibacterial (Embleton et al., 2002) photodynamic therapy. However, erythrosine is already widely used in the dental surgery to visualize dental plaque *in vivo* at doses many fold higher than those used in this study with no apparent detrimental effects on the oral mucosa. Erythrosine-based PDT has also been shown to be non-toxic to eukaryotic cells *in vitro* at PDT doses which are toxic to bacteria (Wood et al., 2006 and unpublished data). Patterson et al. (1990) stated that there seems to be a threshold for PDT effects, where tissue damage seems to be repairable at low doses of the photosensitiser. However, that threshold *in vivo* is not yet known for erythrosine that is irradiated for 15 minutes continuously with a tungsten filament lamp. The only information available is that “related effects should not be an issue if the acceptable daily intake ADI guidelines (0.1 mg/kg body weight/day) for erythrosine that is not irradiated are followed (Ganesan et al., 2011).

As well as the clinical relevance in terms of treatment time, topical irradiation for longer times may also cause burning sensation *in vivo*, as has previously been reported following topical administration of the photosensitiser aminolevulinic acid (Konopka and Goslinski, 2007) for the treatment of cancer. In addition, long duration irradiation regimes for a photosensitiser can cause superficial necrosis, but leaving little scarring and no cumulative toxicity (Moor et al., 2003). However, there seems to be a threshold for PDT effects, where tissue damage seems to be reparable at low light doses (Patterson et al., 1990). The light dose is the product of the irradiation time and intensity of the used light source. In this study the intensity of the light source (tungsten filament lamp) was the same in all groups, whereas the irradiation time was not. Therefore, according to the statement made by Patterson et al., (1990) it would make sense to suggest that there might be a threshold for PDT effects, where tissue damage seems to be reparable for a small irradiation time at a given intensity of the used light source. This time is still unknown for *in vitro* erythrosine-based PDT using a tungsten filament lamp and 220 μ M erythrosine, but it is clear that shorter irradiation times are preferable to longer ones.

The presence of the outliers and extreme outliers, of both phase 1 and phase 2, could have been potentially related to accidental findings (highly unlikely) or to the procedure of plate culturing or to a combination of the above. The reliability of the determination of the total bacterial counts by plate culture has been questioned as it is a retrospective method of bacterial viability (Berney et al., 2007) and its reproducibility is affected by factors such as transport and culture media, temperature, atmosphere and incubation period (Lehtinen et al., 2004). These problems can potentially lead to an underestimation of the total bacterial counts or overestimation in very rare cases. The statistical tests of the present study were repeated in SPSS following removal of the outliers and extreme outliers. There was no difference in the results obtained from this study with or without the presence of outliers and extreme outliers.

This would be of special use in children with special needs who may be unable to carry out adequate brushing due to a physical or medical condition or as a result of severe learning difficulties adequate plaque removal by mechanical techniques would be challenging or unattainable.

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Conflict of Interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of the paper

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Legends

Table 1. Percentage reduction of total bacterial counts in three groups (15 subjects)

Table 2. Percentage reduction of total bacterial counts in six groups (15 subjects)

Fig 1. Flow chart of the study

Fig 2. The intra-oral appliance used in the study

Fig 3. Boxplot of the percentage reduction of the total bacterial counts in three groups (15 subjects) – observation 24 was an extreme outlier

Fig 4. Boxplot of the percentage reduction of the total bacterial counts in six groups (15 subjects) - observations 4, 24 and 28 were extreme outliers

Fig 1. Flow chart of the study

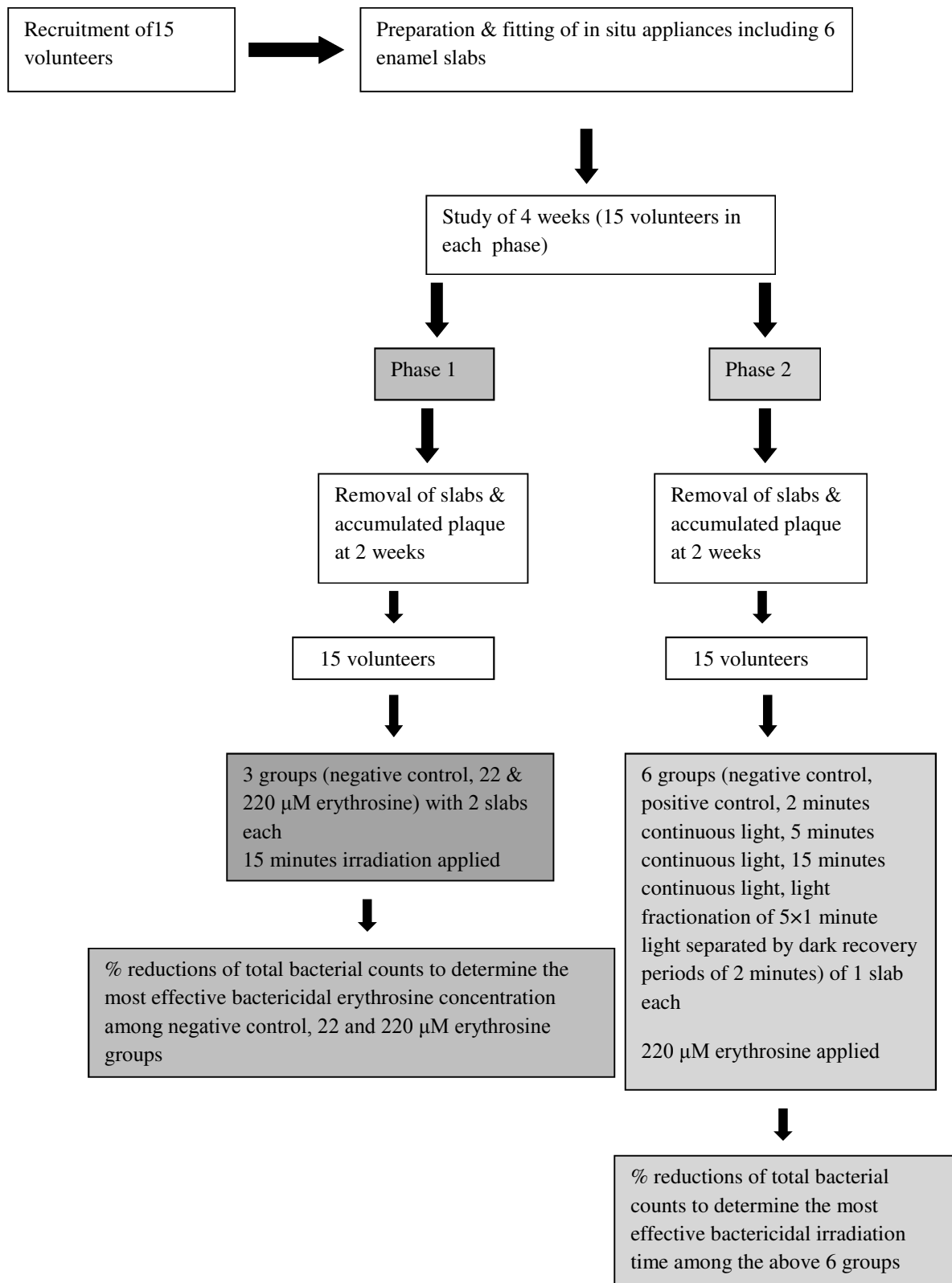


Fig 2. Lateral view of experimental appliance



Table 1. Percentage reduction of total bacterial counts in three groups (15 subjects)

%Reduction of total bacterial counts

Treatment	N	Minimum	Maximum	Mean	Median
No erythrosine, no light	15	.00	.00	.0000	.0000
22 erythrosine, 15 mins light	15	59.24	99.21	86.2808	91.4286
220 erythrosine, 15 mins light	15	73.96	99.66	95.2515	98.2927
Total	45	.00	99.66	60.5108	87.7778

Table 2. Percentage reduction of total bacterial counts in six groups (15 subjects)

%Reduction of total bacterial counts

Treatment	N	Minimum	Maximum	Mean	Median
No light,no erythrosine	15	.00	.00	.0000	.0000
No light,220 erythrosine	15	-13.89	3.85	-1.6035	1.2821
2 mins light,220 erythrosine	15	12.31	90.71	61.1578	63.8462
5 mins light,220 erythrosine	15	37.31	95.49	81.6960	87.9487
5*1 mins light,220 erythrosine	15	51.94	98.57	86.9874	92.8000
15 mins light,220 erythrosine	15	72.78	99.44	94.5454	96.5574
Total	90	-13.89	99.44	53.7972	75.0253

Fig 3. Boxplot of the percentage reduction of the total bacterial counts in three groups (15 subjects) – observation 24 was an extreme outlier

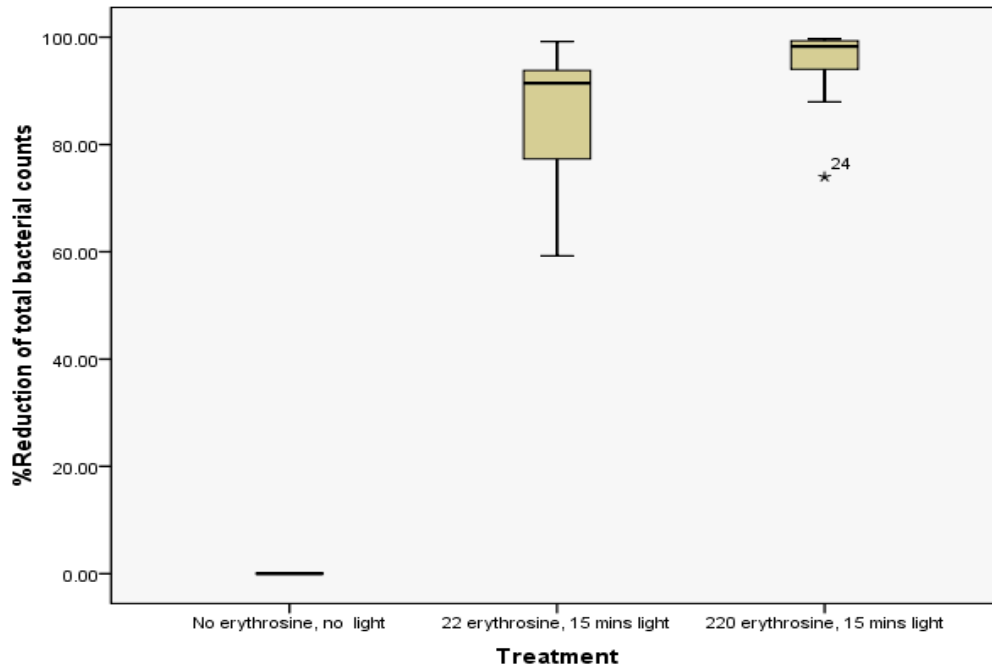


Fig 4. Boxplot of the percentage reduction of the total bacterial counts in six groups (15 subjects) - observations 4, 24 and 28 were extreme outliers

