Original Article

The use of methylene blue as mouthwash in periodontology

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Abstract. The aim of this study is to investigate the cytotoxicity effects of methylene blue on the human gingival fibroblasts cell-lines *in vitro*.

3T3 human gingival fibroblast cell-lines were used to evaluate the cytotoxicity of mouthwashes containing methylene blue and chlorhexidine gluconate. The cultured fibroblasts were divided into two groups which subjected into chlorhexidine gluconate or methylene blue *in vitro*. The cells viability was determined at 30 seconds, 1 minute and 3 minutes of exposure to mouthwashes using by XTT colorimetric assay. Spectrophotometric absorbance was measured at 550 nm using ELISA analyzer. The IC_{50} values were calculated for each time points for methylene blue and chlorhexidine gluconate mouthwashes.

The effect of the methylene blue and chlorhexidine gluconate on the human gingival fibroblast viability was expressed as a percentage of the control groups. Comparison between two groups in different time and concentration values showed that, chlorhexidine gluconate were found to be more cytotoxic on gingival fibroblast than methylene blue. Cell viability exposured to methylene blue in 1% concentration during 3 minutes was 99% and in 100% concentration the cell viability was 88%. The chlorhexidine gluconate at the same conditions was 92% and 18% respectively.

Our study demonstrates that 100% chlorhexidine gluconate (0.2% chlorhexidine gluconate concentration in commercially available products) has much more cytotoxic effect than methylene blue to human gingival fibroblast at clinical use time and different concentrations *in vitro*.

Key words: Methylene blue, chlorhexidine gluconate, cytotoxicity, mouthwash

1. Introduction

Gingivitis and periodontitis are the most common forms of inflammatory diseases in periodontology. Gingivitis is the inflammation of gingiva caused by plaque accumulation (1). Periodontitis can occur if an imbalance between host defense system and the bacterial niche becomes (2). Their primary etiology is bacterial plaque, which can initiate destruction of the gingival tissues and periodontal attachment apparatus (1, 3). The commonly used periodontal therapy is mechanic remove of the plaque or calculus and the use of topically applied antimicrobial agents which can inhibit periodonto-pathogenic bacteria (4). The scaling and root planning procedures using hand, sonic, or ultrasonic instruments is commonly used by dentists to remove calculus. The aim of scaling and root planning is to remove plaque and calculus and to reduce the subgingival bacteria to acceptable levels for preventing clinical inflammation (5). The use of topical antibacterial agents is to reduce bacterial plaque in gingivitis and periodontitis (6).

Antiseptics commonly used in periodontal therapy are; iodine, sodium hypochlorite, chlorhexidine, peroxides, ethyl or isopropyl alcohol, phenolic compounds, quaternary ammonium compounds, etc. Besides, some extracts which produced from plants have been offered as antiplaque agents in current treatment of periodontal and other applications (6).

But there is no adequate data that related with using of these chemicals in clinic (7). One of these agents is methylene blue. Methylene blue, a well-known phenothiazinium dye,is used in photodynamic therapy. This therapy is combination of action of three components; photosensitizer, long wavelength light and molecular oxygen. The application of these

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components leads to convert oxygen molecules to reactive oxygen molecules which can affect the target cells (8). Several studies demonstrated that methylene blue can be used in the treatment of periodontitis (9). Many oral bacterial species are sensitive to photodynamic therapy *in vitro*. Gram positive bacterial and fungal cells have been more susceptible to photosensitizer dyes than gram negatives. Some studies demonstrated that photodynamic therapy can inactivate the bacteria in biofilms (10). Methylene blue affect microorganism and inactivate them without formed of oxygen radicals in dark conditions (11) and also, methylene blue has minor cytotoxicity for the human cells (12).

The use of chlorhexidine for antiseptic therapy is considered as gold standard approach (13). It is known that chlorhexidine is the most used and tested antimicrobial agent in periodontology. Chlorhexidine has cytotoxic effect to many cells including fibroblasts, periodontal ligament cells, alveolar bone cells, and osteoblastic cell line (14,15). Studies reported that chlorhexidine has dose dependent cytotoxic effects on cultured gingival cells (16). Therefore chlorhexidine is used as positive control in this study.

Chlorhexidine has toxic effects not only to bacteria but also to oral tissues. Therefore, chlorhexidine can alter wound healing by applying directly to surgical wounds in the oral cavity (14).

According to these reports in the literature, clinicians should think to use alternative antiseptics that have less cytotoxic effect and wide spectrum antibactericidal properties. Methylene blue is a dye material that commonly used in photodynamic therapy (11). But it can be used as an antiseptic agent in clinical medicine (17). Our literature findings support that there is no adequate data to use methylene blue as a mouthwash in periodontology. Our aim for this investigation is to evaluate the cytotoxic effects of methylene blue on human gingival fibroblast cell-lines *in vitro*. It is the first study that evaluates the methylene blue as a mouthwash and compares its cytotoxicity with a well-known chlorhexidine mouthwash.

2. Materials and methods

This study was to determine the cytotoxicity effect of various concentrations of chemical anti-plaque agents which are commercially available mouth rinses such as chlorhexidine gluconate (0.2% on human cultured gingival fibroblast proliferation.

Chlorhexidine 0.02% 200 ml (2.23nM) was supplied from commercially available product klorhex, Drogsan Chemical Industries, Turkey and Methylene Blue 10 µg/ml 15 ml Bucco Blue (32.2 mM) was supplied from *Koz Pharmaceutical Industry, Turkey.*

2.1. Cell culture

Cell cultures of 3T3 gingival fibroblast were used to evaluate the cytotoxicity of mouthwashes containing methylene blue. İn this study, Chlorhexidine was used as positive control for determination of cytotoxicity level. The cells viability was determined at 30 seconds, 1 and 3 min. of exposure to mouthwashes using by XTT colorimetric assay (Biotium, 3007, Hayward, CA).

Human gingival fibroblast 3T3 cells were obtained from Dr. Fikret Şahin, Ankara University,Faculty of Medicine,Department of Microbiology, Ankara. The cells were cultured in Dulbecco's Minimum Eagle's medium (DMEM) with 10% fetal bovine serum, 100 unit/mL penicillin and 100 mg/L streptomycin. Cultures were maintained at 37^{o}C in a humidified atmosphere of 5% CO_2 and 95% air.

Confluent cells were separated with 3mL (0.25% trypsin and 0.05% EDTA) for 5 minutes, the cells were removed from the flasks by gentle percussion and the enzyme activity was stopped by adding 3 mL Dulbecco's Minimum Eagle's medium with 10% fetal bovine serum and the removed cells were subcultured. The cells were subcultured every 3 days. These cell lines were plated in 96-well plates at $1x10^4$ well density in 10 uL medium and incubated overnight for cell attachment. 3T3 gingival fibroblasts were plated in 96-well culture plates and cultured for 24 h to allow for cell attachment. The medium was removed and the mouthwashes were added to the adherent cell layer in different concentrations for methylene blue and chlorhexidine. Considering the commercially available methylene blue and chlorhexidine concentrations as 100%, the anti-plaque agents were diluted to 1%, 10%, 25%, 50% and 100% with DMEM and filtered by sterile 0.22 µm membrane filters (Merck, Germany).

The cultured fibroblasts were divided into two groups.

- Group 1. Those subjected to chlorhexidine gluconate for positive control
- Group 2. Those subjected to methylene blue

Cells were applied with various concentrations of chlorhexidine gluconate and methylene blue (1%, 10%, 25%, 50% and 100%) for each time point (30 seconds, 1, 3 min) to determine dose and time dependent effect on cell viability. The control group was not subjected to any of the

mouthwashes. In all groups, the viability and proliferation of gingival fibroblasts was measured by XTT colorimetric assay (Biotium, 3007, Hayward, CA) as described by manufacturer instructions. The absorbance of each well at 450 nm was measured. Growth inhibition was compared with untreated controls and analyzed using GraphPad Prism 5 software (San Diego, USA) for IC_{50} determination.

All the experiments were performed for at least four times. The value obtained with the control was thought as indicating 100% viability. Cytotoxicity was sequenced based on cell viability relative to controls. The rate of 90% cells viability assigned slightly cytotoxic, 90-60% cell viability temperately cytotoxic, 59-30% strongly cytotoxic.

2.2. Statistical analysis

The Shapiro-Wilks normality test was used to verify the normality of the data. If the data were normally distributed, the statistical analysis was performed by One-Way ANOVA test using the SPSS 22.0 (IBM SPSS, Chicago, USA) software. *p* value<0.05 was considered statistically significant for all tests. Comparison of cell viability were performed using paired sample t test where normality of data was confirmed using the Shapiro-Wilks normality test.

If data was not normally distributed, groups were compared using Kruskal-Wallis non parametric test which t showed difference between group, Mann Whitney U comparison test was applied.

3. Results

The effect of the mouthwashes on the human gingival fibroblast viability is expressed as a percentage of the control groups. According to XTT assay results, two groups in different time and concentration values showed that, chlorhexidine gluconate were found to be more cytotoxic on gingival fibroblast than methylene blue (Table 1). Viability of different concentration at 30 seconds, 1min, and 3 min exposure of mouthwashes described in Figure 1, Figure 2, Figure 3, Figure 4, and Figure 5.

Effect of methylene blue and chlorhexidine gluconate in different concentration of different time exposures upon gingival fibroblast is showed at Table 1 and Table 2. No statistically significant differences were detected among the groups of methylene blue for viability parameters at any time points (p=0.189). The amounts of viability, were significantly reduced in the groups of chlorhexidine gluconate with the time exposure 0.5-3min. (p=0.46).

Table 1. Effect of methylene blue in different concentration and time exposures upon gingival fibroblast.

concent./viability	0.5min	1 min	3 min
0%	100	100	100
1%	94	83	99
10%	95	82	94
25%	98	79	92
50%	94	76	83
100%	93	75	82

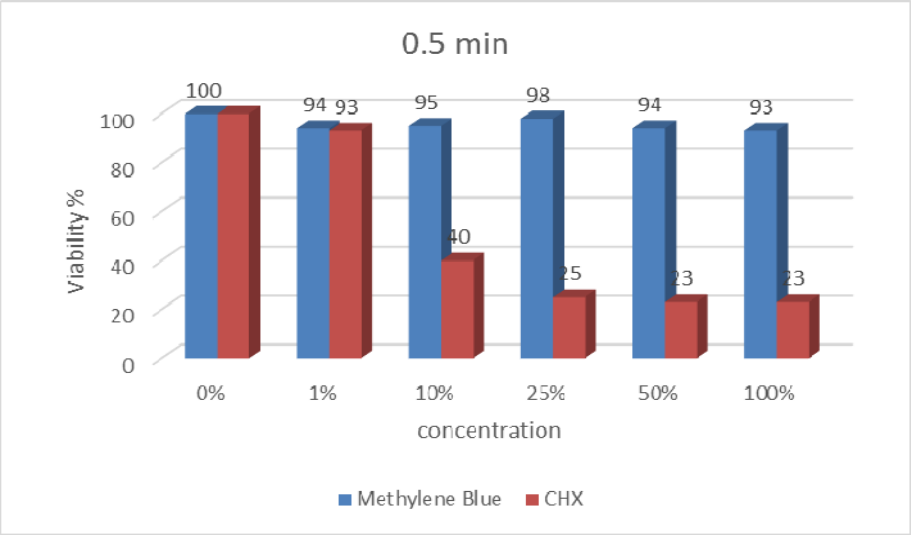

Fig. 1. Viability of different concentration at 0.5 min. exposure of mouthwashes.

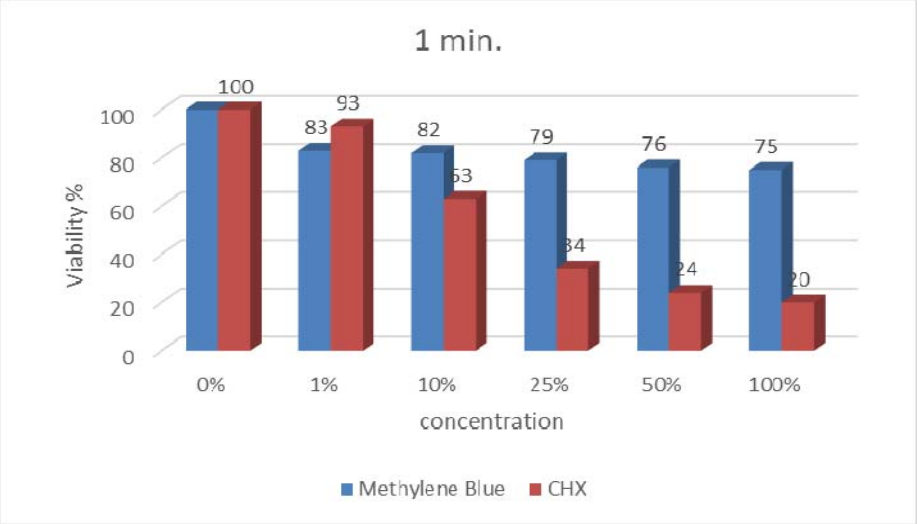

Fig. 2. Viability of different concentration at 1 min. exposure of mouthwashes.

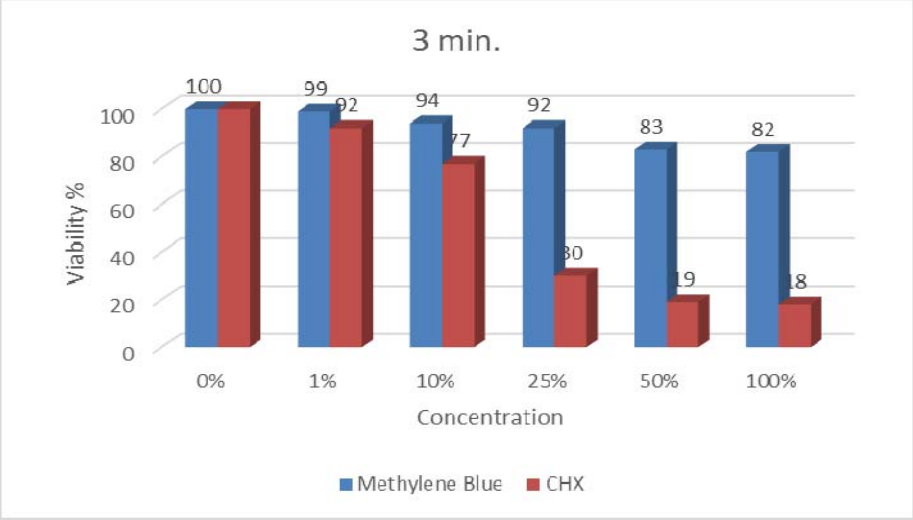

Fig. 3. Viability of different concentration at 3 min. exposure of mouthwashes.

Table 2. Effect of chlorhexidine gluconate in different concentration and time exposures upon gingival fibroblast

concent./viability	0.5 min	1 min	3 min
0%	100	100	100
1%	93	93	92
10%	40	63	77
25%	25	34	30
50%	23	24	19
100%	23	20	18

IC_{50} values for methylene blue and chlorhexidine gluconate was calculated at the time points (0.5, 1, 3 min.) and showed at Table 3. The mouthwashes had different IC_{50} values. At 0.5 min, IC_{50} values for chlorhexidine gluconate calculated as 16 and for methylene blue 1064, at 1min., IC_{50} values for chlorhexidine gluconate calculated as 20 and for methylene blue 199 and at 3 min., IC_{50} values for chlorhexidine gluconate calculated as 20 and for methylene blue 121.

4. Discussion

In a study, the bactericidal effect and the clinical outcomes of methylene blue was evaluated in chronic periodontitis patients. 0.1% sterile methylene blue solution was applied to test sites and microbiological and clinical values evaluated

Table 3. IC_{50} values of chlorhexidine and methylene blue in different time points

Groups	IC50 values		
	0.5 min	1 min	3 min
Chlorhexidine gluconate	16%	20%	20%
Methylene blue	1064%	199%	121%

with the control sites in chronic periodontitis patients. Cocci and facultative organisms were increased and gram negative anaerobes, spirochetes and motile organisms decreased after the irrigation of methylene blue at the end of 14 days comparing to control sites. The redox potential of the methylene blue is determined and found that test sites which were used methylene blue as an irrigating solution has better clinical and microbiological outcomes than control sites which is irrigated with sterile water (9). But the cytotoxicity effect of methylene blue as an irrigation solution in chronic periodontitis patients was not evaluated.

The cytotoxicity effect of methylene blue as a photodynamic therapy dye material is evaluated in a study which the cytotoxicity assay was MTT. Human gingival fibroblast culture was used to evaluate the cytotoxicity in various concentrations and various red light conditions. The results were; at 24 h, the mitochondrial activity evaluated with MTT assay reduced 27% with methylene blue 50 ug/mL and 163.8 j/cm2 red light. This was significantly lower than the controls. But incubation of fibroblasts in dark conditions with 12.5 ug/mL, 25 ug/mL and 50 ug/mL did not show significant decrease in optical density at 24h of evaluation. Also

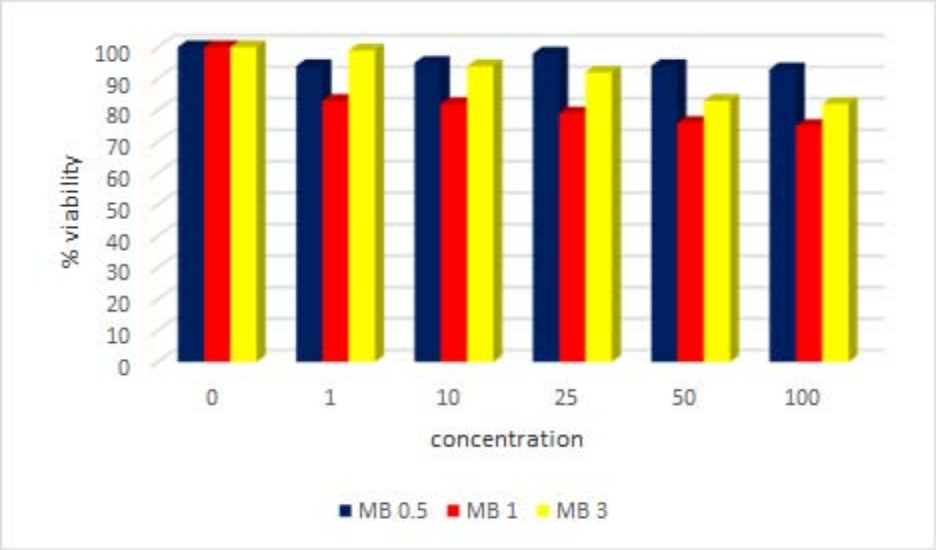

Fig. 4. Viability of different concentration at different time exposures of methylene blue mouthwash.

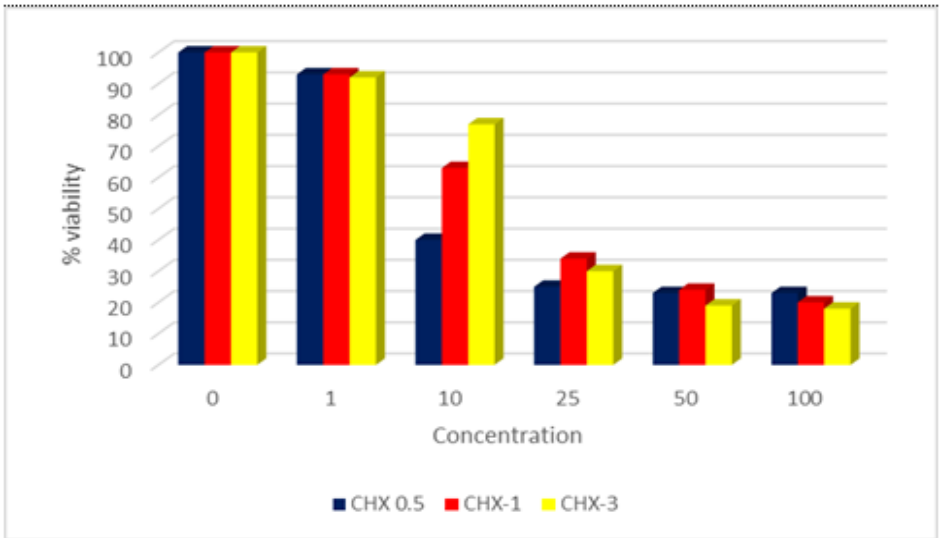

Fig. 5. Viability of different concentration at different time exposures of chlorhexidine gluconate mouthwash.

exposure of fibroblast to 54.6, 109.2 and 163.8 j/cm2 red light in the absence of methylene blue did not significantly induce the cytotoxicity in fibroblasts (12). In this study the used concentrations of methylene blue is far below then we used in our study. We used methylene blue 208 times higher than used in this study. Also the researchers reported that there was no statistically significant reduction in mitochondrial activity of fibroblasts after exposure to light alone or methylene blue alone in the dark. The researchers concluded that the microorganisms are more susceptible to methylene blue than human cells (12). In our literature search, we can not find any study that demonstrates the cytotoxicity effect of methylene blue used as mouthwash in periodontology. In our study we used chlorhexidine as a positive control for cytotoxicity test and evaluated the effect of methylene blue cytotoxicity to human gingival fibroblast cell lines.

In a recent study, the cytotoxicity levels of methylene blue for most endodontic pathogens including, *P. micros, E. Faecalis, F. nuc., P. intermedia, P. gingivalis, P. endodontali*s was measured in 25 ug/ml concentration. Strong toxicy was detected for endodontic pathogens from 70% to 100% after incubation in dark conditions with methylene blue. The survival rates of *P.gingivalis* was %6.7 ±0.1 and *P.intermedia* was %0 in dark conditions treated with methylene blue (25ug/mL). *F.nucleatum* and *E.faecalis* survival rates in dark conditions treated with only 25ug/ml methylene blue were 5.5±0.4 and 94.5±17.6 respectively. But in 30 j/cm^2 red light conditions this rates decreases to 0% and 47±12.1% respectively (11). In this study the concentration used to demonstrate the cytotoxicity effect of methylene blue to endodontic pathogens were far below the concentrations evaluated in our study. This study demonstrates that methylene blue can be used to reduce oral bacteria. But there is no study that evaluates the cytotoxicity effect of methylene blue to oral bacteria when used as mouthwash in periodontology.

Our study findings demonstrate that methylene blue has minor cytotoxic effect upon human gingival fibroblasts. Studies made by other researchers reported that chlorhexidine has much more cytotoxic effects than methylene blue *in vitro* (18). Also methylene blue has anti-bactericidal effect to many oral bacterial species that effective in periodontal destruction (11). At 0.5-minute time point, 1% concentration of methylene blue has 94% viability ratios. This means that there is 6% cell death comparing to controls. At the same time point and concentration, chlorhexidine gluconate has 93% cell viability with the meaning of 7% cell death. But at 100% concentration and at the same time point (30 seconds), the viability of methylene blue still remains at 93% levels with the meaning of only 7% cell death. But the chlorhexidine gluconate viability levels at the same concentration were 23% with the meaning of 77% cell death in 30 seconds. At the time point of 3 min., the methylene blue viability starts with 99% and in 100% concentration ends with 88% cell viability. The chlorhexidine gluconate at the same conditions starts with 92% cell viability and ends with 18% in 100% concentration. With the consideration of 100% chlorhexidine gluconate concentration, this is equal to 0.2% chlorhexidine gluconate concentration in commercially available products, chlorhexidine gluconate has much more cytotoxic effect than methylene blue to human gingival fibroblast at clinical use time and concentration levels *in vitro*.

In periodontology, antiseptics are used widely after the periodontal therapy. The cytotoxic effect of the antiseptics to human gingival cells such as gingival fibroblasts becomes a problem to be solved. Therefore, clinicians tend towards new antiseptics that have less cytotoxicity to gingival cells and wide bactericidal effects. It is known that methylene blue has bactericidal effect to many periodontal pathogens. But according to our knowledge, there is no any information about likely cytotoxic effect of methylene blue as a mouthwash on human gingival cells such has gingival fibroblasts in the literature. This study showed that methylene blue as a mouthwash has slightly cytotoxic effects than commonly used antiplaque agent chlorhexidine gluconate. We can suggest that dentists should prefer methylene blue as an antiplaque mouthwash in periodontology. This is the first study showing that methylene blue could be used as a mouthwash in periodontology. Further studies with high level molecular techniques needed to confirm this result *in vivo* and *in vitro*.

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