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Research Report

Viability test of photodynamic therapy with diode laser waves length 405 nm on BHK-21 fibroblast cells with various irradiation distances

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ABSTRACT

Background: *Photodynamic therapy has now become popular, but its cytotoxic effect is still unclear. In order to be considered suitable for oral cavity therapy, the therapy must not be toxic or cause adverse effects on the target tissue. Viability testing for photodynamic therapy is important to do. Fibroblast cells are often used for testing the toxicity of dentistry because they are the most important cells in the components of the pulp, periodontal ligament, and gingiva.* ***Purpose:*** *To prove the effect of irradiation distance on photodynamic therapy on the viability of BHK-21 fibroblast cells.* ***Methods:*** *Viability test was performed with BHK-21 fibroblast cells placed on a 96 well microplate which was then irradiated with 405 nm photodynamic therapy with varying irradiation distances of 1, 4, 7, 10, 13, and 16 mm. After irradiation, cell viability was tested by MTT assay and ELISA Reader. Data were analyzed using Kolmogorov-Smirnov, Levene's test, Kruskall Wallis, and Tukey HSD.* ***Results:*** *Fibroblast cells with 4 mm irradiation distance have viability over control cells, whereas at irradiation distances 1, 7, 10, 13, and 16 mm have less viability than control cells.* ***Conclusion:*** *Photodynamic therapy 405 nm with 4 mm irradiation distance gives a biostimulation response so that the viability of BHK-21 fibroblast cells increases.*

Keyword*: diode laser; cell viability; fibroblast cells*

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INTRODUCTION

Root canal treatment is a combination of mechanical, chemical and cleaning procedures. Mechanical root canal cleaning combined with chemical solution irrigation is proven to still leave a residual bacteria of about 50% [1]. Photodynamic therapy is one of the additional methods for bacterial inactivation. In photodynamic therapy, there is a reaction between light and photosensitizer in the presence of oxygen. This reaction produces reactive oxygen species and causes oxidative damage to bacterial cells. Photodynamic therapy is one of the optimal therapies in eradicating pathogenic bacteria [5]. In endodontic therapy, photodynamic therapy has proven to be effective in reducing root canal infections in vitro and in vivo [6]. Photodynamic therapy has the special advantage of targeting microorganisms without affecting the *host* [7].In addition, photodynamic therapy also has selective antibacterial ability in low concentrations without causing damage to normal cells. Photodynamic therapy has also proven it can be used for disinfection of endodontic treatment without damaging the cells periapical region [5].In the cytotoxicity test, photodynamic therapy is relatively lower when compared to NaOCl, making it safer for photodynamic networks to be able to inhibit the development of bacterial resistance mechanisms because photodynamic therapy is highly reactive with non-specific organic molecules, so macromolecules in bacterial cells are potential targets [8]. Other advantages of using this photodynamic therapy include being safe for tissue, being able to eradicate bacteria on biofilms, easy to apply and not painful [5].

Photodynamic therapy has now become popular, but its cytotoxic effect is unclear [9]. In order to be considered suitable for oral cavity therapy, the therapy must not be toxic or cause adverse effects on the target tissue. Several *in vitro studies* have shown that photodynamic therapy used to kill bacteria has a cytotoxic effect. In addition, research on the capacity of photodynamic therapy in eliminating bacteria and its side effects on healthy tissue has not been widely studied [10]. In addition, in studies using lasers with a wavelength of 400 nm, many distance variations are used. In the study of Astuti et al., (2016) a distance of 1.5 cm was used. In a study conducted by Masson-Meyers et al., (2015) used a distance of 0.3 cm to 0.5 cm. In a study conducted

Kumar et al., (2016) used a distance of 12 mm. Based on these considerations, this research was conducted using a variation of 1-16 mm distance.

Viability testing for photodynamic therapy is important to do. Viability tests can use animals *in vivo* or cell culture *in vitro* (cytotoxicity). The cell culture used in this experiment was BHK-21 cells from hamster baby kidney fibroblasts. Fibroblasts are often used by researchers for testing the toxicity of dental materials because they are the most important cells in the components of the pulp, periodontal ligament, and gingiva. One method used for testing toxicity by monitoring enzyme activity is the MTT test. MTT is a soluble yellow molecule, which can be used to analyze cellular enzymatic activity. The MTT test is based on the ability of living cells to reduce MTT salt[14]. Based on the above it is considered important to conduct a study of the toxicity test of photodynamic therapy of BHK-21 fibroblast cells with various irradiating distances.

MATERIALS AND METHODS

Type of research conducted is an experimental laboratory. The research design used was *Post Test Control Group Design*. Making BHK-21 fibroblast cell cultures and MTT Assay tests were carried out at the Veterinaria Farma Center (PUSVETMA). The sample in this study was BHK-21 fibroblast cells. In this study used 8 samples for each study group, so that the total sample of research used in this study were 64 samples. In this study there were 8 research groups consisting of, group I (control media), group II (control cells), group III (1 mm irradiation distance), group IV (4 mm irradiation distance), group V (7 mm irradiation distance), group VI (irradiation distance 10 mm), group VII (irradiation distance 13 mm), and group VIII (irradiation distance 16 mm).

The tools used in this study consisted of: CNC Laser length 405 nm, *Centrifuge, Laminar flow,* Microscope, 37incubator°C, *ELISA reader, Shaker,* Bottle *Roux, Microplate 96 well, Multichannel pipette*. While the materials used consist of: Chlorophyll (K-Link liquid chlorophyll) photosensitizer, BHK-21 (fibroblast cells*Hamster Kidney-21),* Media *Eagle, Phosphate Buffer SalinePhosphate Buffer Saline* (PBS),(PBS), *Fetal Bovine SerumFetal Bovine Serum* (FBS) 10%,(FBS) 10%, *Methylliazolydiphenyl-tetrazolium bromide* (MTT), *Dimethylsulfoxide* (DMSO) 50µm, *Trypsine versene.*

The Preparation Stage of BHK-21 Cell Culture begins by inserting frozen fibroblast cell culture into the incubator for 10 minutes at 37˚C until liquid. After that in the *centrifuge* 5 minutes at 1500 rpm. In laminar flow, the supernatant is removed so that the remaining cell deposits at the bottom. The cell deposit is taken and suspended into media *Eagles* and *fetal bovine serum* (FBS) 10%. Media was *Eagles* 36% added to the bottle containing 4 ml serum so that the final result of 40 ml media obtained *Eagles* along with serum. The suspended cell deposit is planted in a bottle *Roux* sterile, then incubated at 37˚C, until the monolayer cells are formed (± 2 days, seen on a microscope). Fibroblast cells were taken from BHK-21 cell culture in the form of *cell-lines* and then implanted in bottles *Roux*. Media on bottles *Roux* containing BHK-21 fibroblast cells are removed and washed with *Phosphate Buffer Saline* 15 ml(PBS) 3-5 times. Bottle is *Roux's* filled with *versps trypsine* 1 ml of. Cells in the bottle will be seen clustered and then homogenized with media *Eagles* 10 ml. Homogeneous cells were inserted into a *microplate* 96 *well* with a density of $2x10^5$ cells / ml as much as 50 µl and incubated for 24 hours.

The Stages of Treatment begin with *microplate* containing fibroblasts which have been incubated, under a light microscope observed to ensure that fibroblasts planted in each *well are* sufficient for research. Before fibroblast cells are given chlorophyll photosensitizer, the photosensitizer is diluted first so that the concentration becomes 0.2 mg / ml. Fibroblasts cells that had been distributed in *well were* divided into 8 treatment groups, namely group I as a negative control without shining, groups II through VII as a research group containing fibroblast cells were given chlorophyll photosensitizer of 0.05 ml and allowed to stand for 7 seconds. Fibroblast cells are then irradiated with photodynamic therapy and chlorophyll photosensitizer with irradiation distance of 1, 4, 7, 10, 13 and 16 mm for 75 seconds. Each treatment has 8 replications which are then planted in *wells*.

After the treatment is finished, the media which has been irradiated in the *microplate is* discarded then washed with PBS. MTT (tetrazolium salt) was dissolved in PBS 5 mg / ml then MTT was dropped on each *well* as much as 10 µl. Then incubated again in an incubator for 2-4 hours at 37˚C. DMSO (*Dimethylsulfoxide) was* added as much as 50 µl to each *well* and vibrated with a *shaker* for 5 minutes until the formazan crystals dissolved. Then, readings *optical density* with *Elisa reader* at a wavelength of 620 nm. The more concentrated the color, the higher the absorbance value and the more number of living fibroblast cells. Living fibroblasts will turn blue, while dead cells will not form blue.

Data obtained from the results of readings *Elisa Reader* then calculated the percentage of living cells. Before testing to analyze the results of *optical density* formazan between groups, it is necessary to test the distribution of samples with the normality *Shaphiro-Wilktest* and sample homogeneity tests with the levene test, if the data is not homogeneous it is necessary to test the significance through the analysis *Kruskall Wallis.* The data obtained were then tabulated and analyzed statistically using the *One Way ANOVA test*. Followed by a different test between groups using *Tukey HSD.*

RESULT

This study aims to determine the viability of fibroblast cells that have been irradiated with photodynamic therapy with various irradiation distances. The method used is *MTT Assay*. In this study consisted of 8 treatment groups consisting of media control, cell control, irradiation with

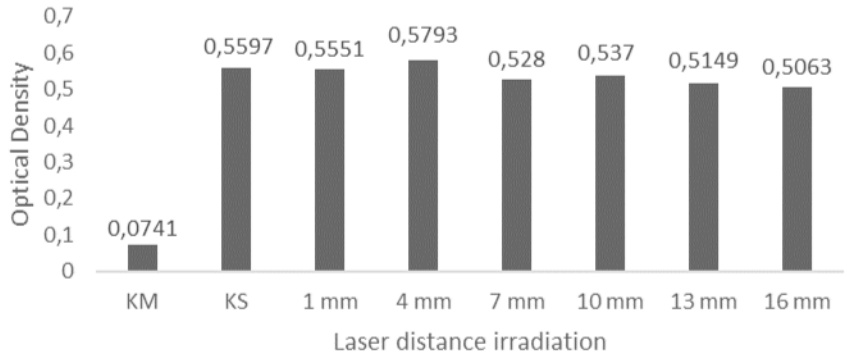

Figure 1. Optical density values of BHK-21 fibroblast cells in the control and treatment groups.

Table 1. The average value of optical density, standard deviation, the percentage of living cells, and the percentage of dead cells in each treatment group

Treatment Group	$\bar{x}$	SD	% Living cells	% Dead cells	N
Control Cell	0.5597	±0.188	100%	0%	8
Control Media	0.0741	±0.051	0%	100%	8
1 mm	0.5551	±0.294	99.05%	0.95%	8
4 mm	0.5793	±0.186	104.3%	-4.03%	8
7 mm	0.5280	±0.188	93.47%	6.53%	8
10 mm	0.5370	±0.242	95.33%	4.67%	8
13 mm	0.5149	±0.191	90.78%	9.22%	8
16 mm	0.5063	±0.176	89.01%	10.99%	8

Notes: $\bar{x}$: The average optical density value; SD: Standard deviation / standard deviation; N: number of groups per treatment.

a distance of 1 mm, 4 mm, 7 mm, 10 mm, 13 mm, and 16 mm. Each treatment group was replicated 8 times. Figure 1 is the reading of the results using the *ELISA reader*.

Data measure the *optical density* using *Elisa reader.* Normality test using the Kolmogorov-Smirnov test to see whether the data obtained are normally distributed. Data is said to be normally distributed if p> 0.05. In each treatment group, the value of p> 0.05 was obtained, meaning that the data obtained were normally distributed. Then proceed with *Levene's test* to find out the homogeneity of the data as a condition of significance test using ANOVA. The results obtained p = 0.000 (p> 0.05) which shows the data are not homogeneous. Therefore, the significance test was carried out using a nonparametric test through the analysis *Kruskall Wallis* and the results obtained p = 0.000 (p> 0.05) which showed a significant difference in the treatment group. Furthermore, to find out the differences in significance between treatments to the control group, the *Post-Hoc comparison test was* used using *Tukey HSD*.

The results of the *Tukey HSD test* in the table show that there are significant differences between the cell control group and the media control group. However, there was no significant difference between the cell group and the treatment group with irradiation distances of 1, 4, 7, 10, 13, and 16 mm. At 1 mm distance laser irradiation found significant differences with media control, laser irradiation at a distance of 4 mm and 10 mm laser irradiation. In the 4 mm distance laser irradiation found a significant difference with media control and 7 mm laser irradiation. In the 7 mm distance laser irradiation, a significant difference was obtained with media control and a 10 mm laser beam irradiation. In the laser illumination of 10 mm distance found significant differences with media control and laser illumination of the distance of 7 mm. In the laser irradiation distance of 13 mm and 16 mm found significant differences only with media control.

DISCUSSION

Photodynamic therapy is a device that usually produces electromagnetic radiation that is relatively uniform in wavelength, phase, and polarization[15]. When a photon is absorbed by the cell, the photon will continue its energy to the cell. The photon energy level determines the type of energy transfer that occurs [16]. The effects of biostimulation and inhibition of photodynamic therapy are regulated by Arndt-Schulz law. According to this law, low doses will increase physiological processes, and strong stimulation will inhibit physiological activity. Low-level photodynamic therapy does not cause an increase in temperature in the tissue, but produces its effect from the effects of biostimulation in the tissue. Photodynamic therapy with low energy levels will not cut or damage tissue, but will cause biostimulation

and biomodulation effects. Enhanced biostimulation with photodynamic therapy has been reported to induce changes in intracellular metabolism, resulting in faster cell division, rate of proliferation, migration of fibroblasts and rapid matrix production[17].

Viability testing for photodynamic therapy is important to know the right dose so that cells can proliferate optimally. The cell culture used in this experiment was BHK-21 cells from hamster baby kidney fibroblasts. Fibroblasts are often used by researchers for testing the toxicity of dental materials because they are the most important cells in the components of the pulp, periodontal ligament, and gingiva. The method used in the study is the MTT *assay*. The MTT test is based on the ability of living cells to reduce MTTsalt [14].

From the research that has been done it can be seen that the viability of BHK-21 fibroblast cells in all groups is above 80%. According to Telli, an ingredient can be said to be non-toxic if the percentage of living cells is more than 50% after living cells have been exposed to a substance. The lowest viability was found in irradiation with a distance of 16 mm with a value of 89.01%, while the highest viability was obtained in irradiation with a distance of 4 mm with a value of 104.03%. From the research that has been done, it can be said that laser exposure with this distance is not toxic [18].

At distances of 7 mm, 10 mm, 13 mm and 16 mm, laser irradiation has no effect on the viability of fibroblast cells. This can happen because the number of photons that are chopped by the chromophore is not enough to stimulate cells. Based on the Anrdt-Schultz curve, the above condition is a state from point A to B (Figure 2.2). According to the Arndt-Schultz law weak stimulation can stimulate biological activity, moderate stimulation strengthens biological activity, strong stimulation can suppress biological activity, and very strong stimulation can stop biological activity [16].

In the group with a 4 mm irradiation distance, there was an increase in BHK-21 fibroblast cells by 4.09%, although when compared with the control group there was no significant difference. Based on the Anrdt-Schultz curve, the above condition is a state from point C to D (Figure 2.2). That means that at that distance biostimulation occurs with the right amount of energy. The existence of enough energy will cause the acceleration of electron transport resulting in a biostimulation reaction. The acceleration of the electron transport reaction will cause an increase in ATP production. Increasing the amount of ATP will accelerate mitosis of cells [19]. Laser-induced biostimulation will increase cell proliferation through the release of *growth factor* [20]. *Growth factors* play a role in controlling collagen breakdown, recruitment and formation of new fibroblasts, formation of new collagen and other matrix components, and the formation of new blood vessels. In research that has been done giving laser therapy proven to increase the production of bFGF (*basic fibroblasts growth factor)*. bFGF stimulates the proliferation of all types of cells involved in the process of wound healing both *in vitro* and *in vivo*. In addition, bFGF also has an important role in cell migration [21].

In the group with a 1 mm irradiation distance, BHK-21 fibroblast cell death was 0.95%, even if compared with the control group there was no significant difference. This means that at that distance bioinhibition occurs in the presence of excess energy. Based on the Anrdt-Schultz curve, the above condition is a state from point D to E (Figure 2.2). This is according to the Arndt-Schultz law. Strong stimulation can suppress biological activity [16]. The bioinhibition effect occurs because chromophores absorb excess photons. This resulted in NO and ROS production increasing by [19]. Cytochrome c oxidase (Cox) activity can be inhibited by nitric oxide (NO). Cox inhibition can be through direct competition between NO and O2 to reduce the *binuclear center* CuB / a3from cytochrome c oxidase, or vice versa. Photodynamic therapy can also cause redox shifts from cells towards greater oxidation. In addition, photodynamic therapy can also increase the formation of ROS [15]. Increased free radicals can cause tissue damage known as oxidative stress. If cells experience oxidative stress, cells will more easily experience death [22].

CONCLUSION

405 nm photodynamic therapy with a 4 mm irradiation distance increases the viability of BHK-21 fibroblast cells.

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