In-vitro Evaluation of Anticancer Activity of Rhodamine-640 perchlorate on Rhabdomyosarcoma cell line

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Abstract

Photodynamic therapy (PDT) is a well-recognized treatment modality for oncological and non-oncological diseases. In this current research Rhodamine (Rh-640 perchlorate), a second-generation photosensitizer, mediated photodynamic effects are evaluated. Different controlling parameters i.e., optimum intracellular drug concentration, light dose response on lower scale is investigated on in-vitro human Rhabdomyosarcoma cancer cell line. Diode laser 630 nm was used as a light source and three treatment arms were selected for photodynamic efficacy evaluation. An optimum incubation time of 3 hours was found for Rh-640 mediated exposure of RD culture. Effective intracellular drug accumulation was optimized for culture when administered with different concentrations (5, 10, 20, 30, 50, 70, and 100) μM. Photodynamic action of Rh-640 at different light doses (2, 5, 15) J/cm^2 was evaluated. It was found that post incubation with optimized parameters Rhodamine 640 mediated photodynamic therapy showed light dose dependent cytotoxicity i.e., (85% $_{(2\ J/cm^2)}$, 80 %$_{(\ 5\ J/cm^2)}$, 69 %$_{(\ 15\ J/cm^2)}$. Current research work suggests the potential photodynamic action of Rh-640 and may be evaluated its efficacy on other cancer cell lines.

Key words: *Photodynamic Therapy (PDT), Rhodamine 640 perchlorate (Rh-640 perchlorate), Cell Culture, Photosensitizer (PS)*

1.1 Introduction

The first cell death by light interaction was reported by Oscar Rabb, a German[1]. PDT is a novel cancer treatment modality that uses light interaction with cells, which results in a cytotoxic generation of reactive oxygen species for cancer cell damage. This includes the exposure of cancer cells to a PS and light of appropriate wavelength [2, 3].The Reactive oxygen species (ROS) mediates the oxygen dependency in PDT that is singlet oxygen responsible for most photodynamic processes in biological systems. Cellular damages are mediated when a PS is activated; it goes to an excited triplet state resulting in two kinds of reactions. A direct reaction with the molecular membrane, to form radical cation or anion is the type 1

reaction. In type 2, a singlet oxygen production from molecular ground state oxygen due to energy transfer from triplet sate of PS to the molecular oxygen. ROS is effective only near its generation site due to its short half-life [4].

There may be apoptosis, necrosis, or damage to organelles as a result of PS localization and oxygen availability. These pathways can cause cell death simultaneously and can coexist according to physiological conditions. Vital cellular components can be damaged by applied light dose in PDT. The cellular component like mitochondria is the localizing site for Photosensitizing components but with certain initializing stressive oxygen production to undergo apoptosis or loss of functioning. If lower doses are used, it will bring apoptotic cell death, and alternatively, for higher doses, it will be necrotic [5].

In PDT, results depend on the interaction of PS with the target cancer/tumor cells due to its localizing ability within organelles of cells as lysosomes, endoplasmic reticulum, Golgi apparatus, plasma membrane, and mitochondria[6]. The structure of PS especially ionic charge -4 anionic to +4 cationic, degree of asymmetry and hydrophobicity determines its intracellular localization. Diffusion of PS across the plasma membrane is seen by its water-hating nature and two or fewer negative charges. Compared to the less hydrophobic PS, with less than two negative charges are difficult to diffuse across the plasma membrane due to being highly polar. Hydrophobic PS accumulates more in cells even in low concentrations. The subcellular localization determines the type of light damage to cells. This results in choosing the PS precisely for each modality[7, 8]. PDT has been investigated for stem cells for the purging of tumors in bone marrow transplants. The photosensitizer used was merocyanine 540. Till now, normal cell working and anti-tumor effect has been a contest to achieve through PDT[9].

In oncological disease, RD is a commonly occurring childhood cancer of soft tissues. Half of the soft tissue sarcomas is RD which is a high-grade neoplasmic malignancy[10]. After neoplasm and Wilm's tumor, it takes third place of being solid tumor at the childhood stage. Out of all occurring cases of pediatric malignancies, 65% are with children ages less than six years. In younger children, RMS(Rhabdomayosarcomas) of the head and neck are common [11]. For both metastatic and advanced RMS there are patients with no improvements in the outcome of clinical therapies in the last 4 decades, so it need trials preclinical and clinically to eradicate theses RMS[12].

Different derivatives of Rhodamine123 have served for damaging cancer cells in PDT due to their high absorption coefficient, high fluorescence quantum yield, and photostability [13]. It is worth mentioning that no one before our work has used a high quantum yield of PS Rh-640 perchlorate for RD cell line. The present study is the first attempt to investigate the concentration, light dose-dependency, and photodynamic outcome of Rh-640 perchlorate as a Photosensitizer which is a Rhodamine class derivative. The purpose is to produce and analyze such a combination of PS and light dose which shows preferred localization as well as uptake by cancerous cells using low light dose[14]. It is to determine the cancer cell viability at optimal concentration, time, and a comparative study of light dose interaction at lower light doses. The phototoxicity of Rh-640 perchlorate will let us suggest that it helps eradicate RD cancer. After obtaining the best results of its lower light PDT outcome, we have investigated its efficacy as a Photosensitizer in RD cell line.

2.0 Materials and Methods

2.1 Cell culture

RD cells were collected from the National Institute of Health (NIH), Islamabad. Cultured in Minimum Essential Medium (MEM) (with Hank's salt together containing 10% Fetal Bovine Serum (FBS), 2mM L-glutamine) along with some essential antibodies (neomycin, penicillin, streptomycin) and non-essential amino acids were incubated for 24 hours to let them properly attached to the substratum. The cells were sub cultured twice a week and maintained at 37 ^{0}C (also provided by a moist environment) as a sub-confluent monolayer in a 25 cm^2 tissue culture plastic flask. Cell culture was harvested using 0.25% trypsin when 75-80% confluence was reached. Every time sub-culturing was done within a biosafety cabinet.

2.2 Photosensitizer

Rh 640 perchlorate was purchased from "Sigma Chemical Co.". The stock solution was prepared with 1.692 mM molarity in Ethanol (99.7% pure), wrapped in aluminum foil, and kept in dark due to its light sensitivity. Varying concentrations of Rh 640 perchlorate were prepared from this stock solution ranging from 0-100 µM. Best results were obtained by using freshly prepared solutions in the experiment by making dilutions from stock solution, with MEM minimum essential medium (serum-free).

2.3 Uptake time of Rh 640 perchlorate by RD cells

A 96 well microliter (flat-bottomed) plate was used to incubate 1×10^5 RD cell/well with Rh-640 perchlorate concentration of 5 µM & 50 µM for 6 hours at 37 ^{0}C. The cellular absorption of Rh-640 perchlorate by RD cells was measured using microwell plate reader ELX×800). The reading was taken every 30 minutes up to 6 hours. The optimal incubation time for PDT was observed at the highest absorption. All results were demonstrated as mean absorbance $\pm\sigma$ (n=3) for low and high concentrations of PS. For each data, while analyzing the results, we will plot the two-time series in both high and low concentrations.

2.4 Dark Cytotoxicity

For performing this experiment RD cells were cultured and seeded (1×10^5 RD cell/well) into 96 well plates, incubated by Rh 640 perchlorate with its varying concentrations from 0-400 µM at 37 ^{0}C for 3 hours. The cytotoxic assay was performed using MTT assay (Micro culture tetrazolium assay 3-(4, 5-dimethyldiazol-2-)-2-5-diphenyltetrazolium bromide) and then extracting solution (containing isopropanol and DMSO 2.5 ml per well) was used to find out the number of viable cells and the 96 well plates were read by micro well plate reader. The percentage of viability in cell population was found using the formula:

$$\%\text{viability} = \frac{\text{Mean Ab}_t}{\text{Mean Ab}_{com}} \text{x}100$$

Where Mean Ab_t is the mean absorbance in the treated cells and Mean Ab_{com} is the mean absorbance of the controlled cells which are not exposed to light.

2.5 Light irradiation

In our experiment, without incubating, the PS RD cells are irradiated with laser light from a semiconductor diode laser (LPT-630/675- BIOSPEC, Russia) at λ = 630 nm. An optical fiber (BIOSPEC, TF-D, Russia) transmitted this light through the clear bottom of the plates to the cells in 6 mm diameter of well. The light dose range from 0-15 J/cm^2 (3 wells/dose) and after the light exposure MEM was removed

and 100-200 μℓ fresh MEM 10% FBS added to each well and plate kept in an incubator. After 2 to 3 hours MTT assay was performed to find out phototoxicity.

2.6 Incubating PS and exposure of light giving PDT results

Our experiment was completed in 2 steps. In the first step, three of the 96 well plates were cultured with RD cells (1×10^5 RD cell/well) and varying concentrations of Rh 640 perchlorate were incubated for the optimum incubation time which was found in the above experiment of uptake time of Rh-640 perchlorate by RD cells. In the second step, light treatment was given in 3 treatment arms. First, the light dose of 2 J/cm^2 was given to one of the plates then, in the second treatment arm, a light dose of 5 J/cm^2 was given to the second plate. In the third treatment arm, 3rd plate was treated with a light dose of 15 J/cm^2. After each light exposure, at the end of treatment arms, a viability assay MTT was performed to find out how much toxicity has been produced in cells. Each experiment was done at least 3 times to confirm data.

3.0 Results and discussion

In the present study, first the optimal parameters such as incubation time of PS, concentration of PS and light doses were found out. The time a PS requires to be localized maximum in RD cancer cells is the optimum uptake time. Thus, accumulation of PS is executed once for low concentration and then for high concentration, as shown in Figure 1-2 below (for 5 μM concentration and also for 50 μM in the RD cell line). Figure 1 demonstrates the incubated time duration of Rh-640 perchlorate for 5 μM. The extreme absorbance corresponds to the optimum uptake time of Rh-640 perchlorate. While the first 2 hours showed an ascending cellular uptake in which absorbance kept on increasing with time interval and at 180 minutes or 3 hours of incubation it indicated maximum absorbance. After that a continued descending pattern was observed while the time for incubation was increasing. At this stage PS started excreting out of cells, which evaluates the capability of cells to absorb PS for assessing the efficacy standards[15]. From figure 2, it can be seen easily that there is the same uptake of Rh640 perchlorate by RD cells regardless a different concentration of 50 μM was chosen. At 3 hours both concentrations (5 μM and 50 μM) showed the optimum uptake time of Rh-640 perchlorate because at this time there is enough PS accretion to produce a toxic effect upon laser light irradiation[16]. Figure 3 show that two different incubated doses have maximum peaks at highest absorbance which reveals the optimum incubation time to be 3 hours. As Rhodamine123 class of derivatives are cationic molecules which concentrate in mitochondria by mitochondrial transmembrane potential ($\Delta\Psi_m$). RD cells are carcinomas of soft tissues and Rhodamine is selectively toxic towards carcinoma cells. Higher accumulation of PS at 3 hours of time may be attributed to higher transmembrane potential that increases mitochondrial outer membrane permeability. This affect may result in release of cytochrome C that impacts the mitochondrial function and initiates apoptosis .The selective killing of RD cells with higher concentration and PS uptake may be correlated with this affect[7, 17-19].

Figure 4 depicts various concentrations ranging from 5 μM to 100 μM were selected and their relative absorbance in RD cells was calculated. It was to investigate the cytotoxic effect of PS and the impact of increasing PS concentrations. This explains the damage caused by the photosensitizer Rh-640 perchlorate in RD cells without light irradiation.

Figure 5 illustrates that PS has a low dark cytotoxicity, as Rh-640 perchlorate demonstrates good viability at 5 μM concentration. Reason behind it might be the survival of cancer cells that is much significant at low doses, resulting in less damage to cancer cells. According to this data Rh-640 perchlorate is less dark

cytotoxic. It demonstrates that PS without light irradiation causes slight cell death, with cell viability remaining at roughly 100%.

The optimized concentration is 100 μM. Rh-640 perchlorate remains nearly nontoxic to cells without light irradiation. For enough cell death of RD cells, light irradiation is required. It is worth noting that the cytotoxic impact of the utilized photosensitizer is also dependent on the kind of cell line. A PS accumulates in mitochondria, potentially inducing apoptosis via the mitochondrial route. Hence, cell survival decreases [20-23]. More precisely, larger doses and longer uptakes of PS affect the viability[24].

Figure 6 depicts varied light dose concentrations in J/cm^2, with the bars representing percent viability values at light doses ranging from 2 to 15 J/cm^2. This will allow us to determine how damaging the light dose (in the low dose range) is for the RD cell line, as well as how it will behave in the absence of a PS. The irradiation range is set between 2 J/cm^2 and 15 J/cm^2, with a high viability persistence of up to 95%. In this range, the viability results are fairly similar to previously published studies on phototoxicity of non-ALA treated Rhabdomyosarcoma cells[25]. The cell death at a high dose is the result of necrosis which increases by increasing further light and drug doses. Thus, light alone does not produce enough cell death. The use of PS is necessary to reduce cell viability. The increase in percentage viability might be accounted for enhanced cell division resulting from the accelerated mitochondrial activity on light irradiation [26].

The above results demonstrate that light doses of 2 J/cm^2 to 15 J/cm^2 may be suitable for PDT corresponding to the viability nearly 100%. To treat RD cells only light is less phototoxic. This makes Rh-640 perchlorate, an essential PS to kill RD cells.

The bar chart in figure 7 is the therapeutic outcome of RD cancer cells when incubated with Rh-640 perchlorate. The laser doses of 2,5,15 J/cm^2 are used. Different 3 light doses are chosen which are irradiated on seven different PS concentrations (0-100 μM). It also includes non-treated cells labeled as control.

The light dose of 2 J/cm^2 was selected for the first treatment arm. The effect of this light dose is shown in figure 7. It demonstrates how much toxicity is produced using light dose of 2 J/cm^2 by varying concentrations of PS from 5 μM to 100 μM. By increasing concentration while giving the same light dose increases the toxic effect. At 70 μM and 100 μM, the behavior is essentially same. When cells are exposed to 100 μM, their viability drops by 85% PS chemically conjugates with its target receptors that include a variety of cell surface receptors. Direct phototoxicity causes irreversible photo damage in some organelles and membranes in PDT[27].

The PDT effect at 5 J/cm^2 is shown in figure 8. The experiment was repeated by keeping the concentrations of PS same but this time at 5 J/cm^2. This also shows a gradual decrease in percentage viability. PS is inducing damage together with an applied light dose of 5 J/cm^2. In the end at 100 μM, the viability is 80% that shows a 5% increase in toxicity.

The 3rd treatment section is at 15 J/cm^2 with varying concentrations of PS. Its bar chart is shown in figure 9, which demonstrates that the efficacy of PDT increases by increasing the concentration of Rh-640 perchlorate at light dose of 15 J/cm^2. This time 16% drop in viability (compared to the previous light dose) occurs at 100 μM concentration of Rh-640 perchlorate.

The comparative PDT effect at 2 J/cm^2, 5 J/cm^2, and 15 J/cm^2 and at different concentrations is demonstrated in figure 10. By comparing different doses, we suggest that the best PDT outcome is at 15 J/cm^2. Different concentrations of PS are used which shows that the viability of cells decreases with

increasing concentration of light dose. The bars shown in Fig 10 demonstrate the experimental values of viability; it also includes the control or non-treated cells. This is a relative comparison for analyzing the effect of PS and light dose to the RD cells. Therapeutic outcome at low doses of drug and light is not much effective in reducing cell viability. The dose of light at 15 J/cm^2 and the concentration of PS at 100 µM produces enough toxicity. It's because of the high quantum yield of Rh 640 perchlorate at high concentrations, which causes cell damage due to the fast rate of singlet oxygen formation. Because haemoglobin and myoglobin absorb less than 600 nm and water over 1000 nm, the optical window is restricted within this range[28]. Poor phototoxicity and low yield of triplet state is observed by Rhodamine derivatives. To overcome this their macro cycle is combined with halogens like heavy metals that enhance the spin-orbit coupling, singlet oxygen quantum yields as well as, triplet lifetime up to 10-fold. From all other Rhodamine derivatives, Rh-640 perchlorate (nearly similar to Rhodamine 101) shows the highest quantum[1, 29]. This effect is amplified in Rh 640 perchlorate, which is based on Xanthene and contains chlorine in its macrostructure, making it hazardous at light exposures (0-15 J/cm^2). The vitality of cells diminishes with increasing quantities of light dosage and PS.

Figure 11 shows that, when compared to other light doses, the 100 µM concentration at 15 J/cm^2 may prove to be more effective in the PDT of RD cell line. When RD cells are not treated with Rh-640 perchlorate, their cellular viability is found to be high. The viability of the cells decreases from 100% to 69% after treatment with PS concentration of 100 µM.

4.0 Conclusions

In this present research the analysis shows that neither Rh-640 perchlorate nor laser light alone can cause a cytotoxic response that kills RD cells when administered independently. PDT caused cell death, and its efficacy has been observed by evaluating different parameters such as optimal absorption time, dark cytotoxicity, and comparative dose-response characteristics at low light levels. It has a noticeable influence on the proliferation of cancer cells. Up to a sufficiently high PS concentrations, the cytotoxic effect is reduced. This indicates that Rh-640 perchlorate is the optimum photosensitizer because it has no dark toxicity and a high quantum yield [30]. Even at 400 µM concentrations, there's no evidence of dark cytotoxicity. The phototoxic effect of laser light (630 nm and 300 mW /cm^2) is also minimal, with no discernible drop in cell viability in the 2 J/cm^2 to 5 J/cm^2 range. Irradiating with higher light dose of 15 J/cm^2 and higher PS concentrations of 100 µM results in an effective decline in viability of up to 69 percent. It is concluded that Rh-640 perchlorate may be used for further photodynamic evaluation.

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Competing interests: I declare that the authors have no competing interests as defined by Springer, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

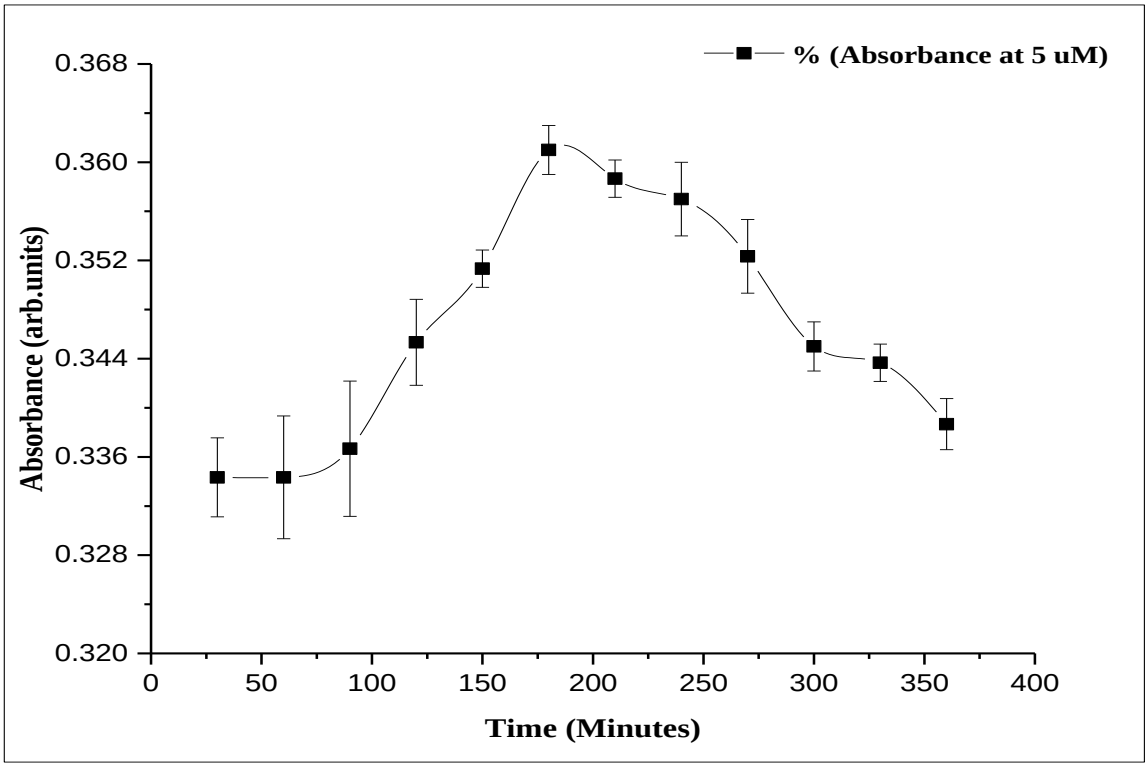

Figure 1: Time vs. absorbance of RD cells incubated with Rh-640 perchlorate at 5 µM concentration.

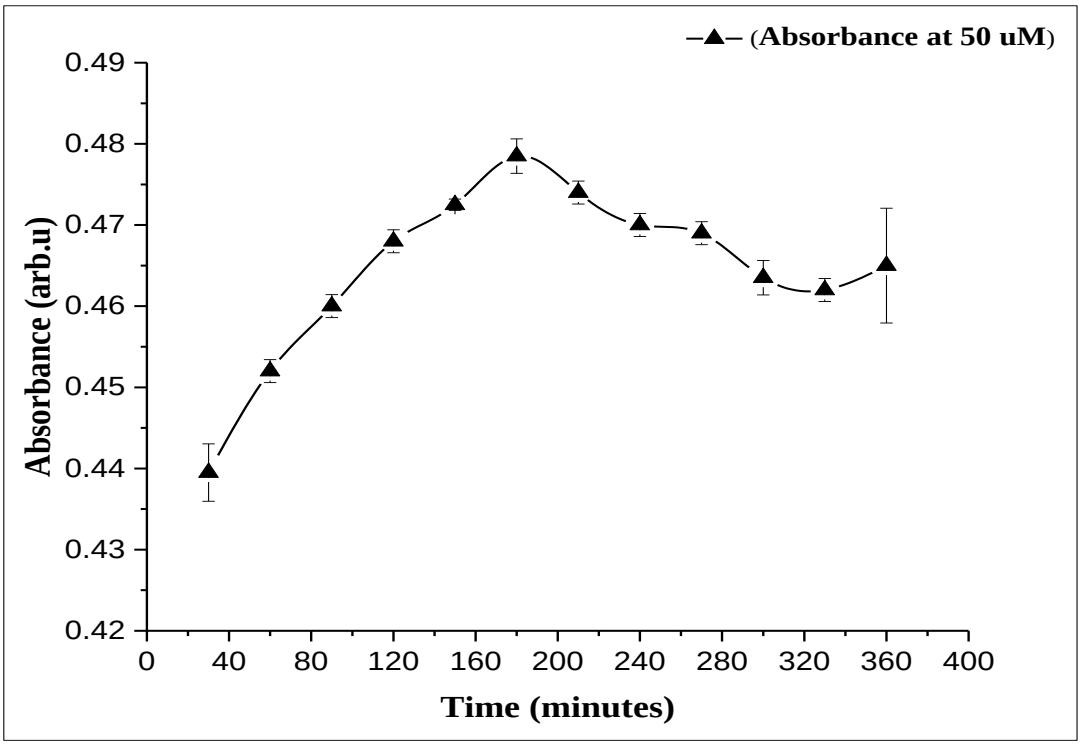

Figure 2: Time vs. absorbance of RD cells incubated with Rh-640 perchlorate at 50 µM concentration.

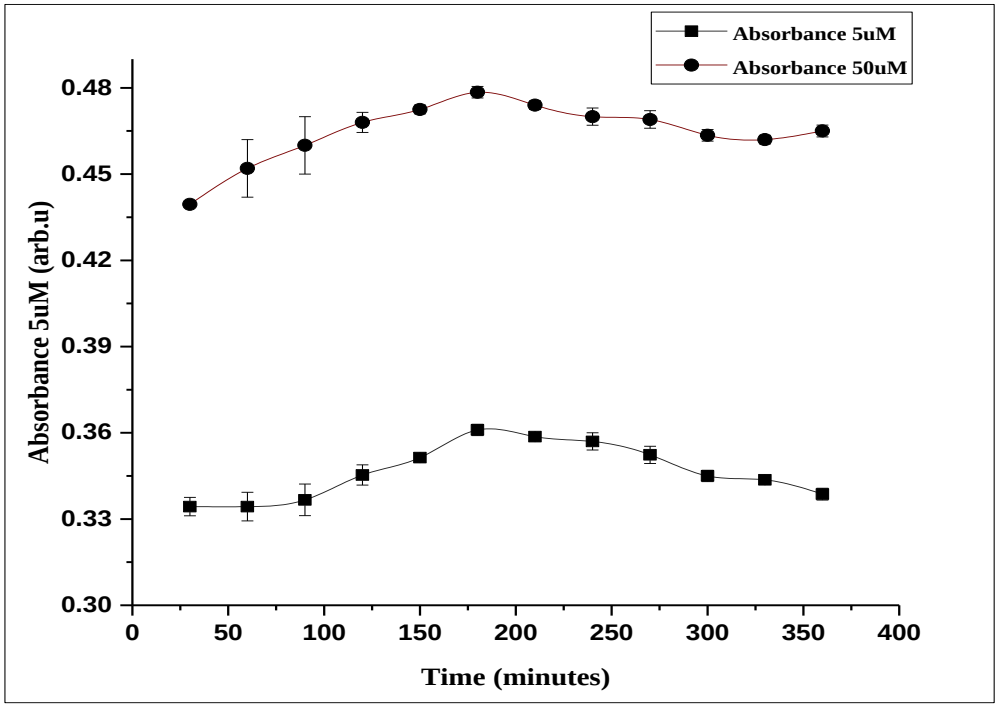

Figure 3: Time vs. absorbance of RD cells incubated with Rh-640 perchlorate at 5 µM and 50 µM concentration.

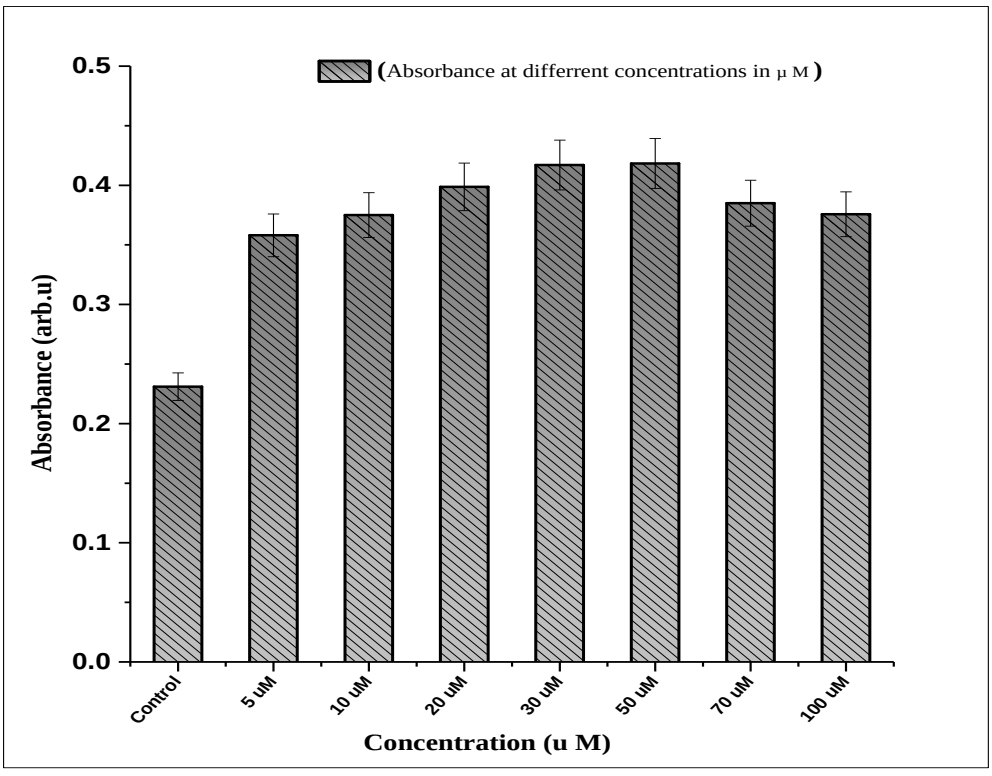

Figure 4: concentrations vs. absorbance of RD cells incubated with Rh-640 perchlorate incubated at the optimum time.

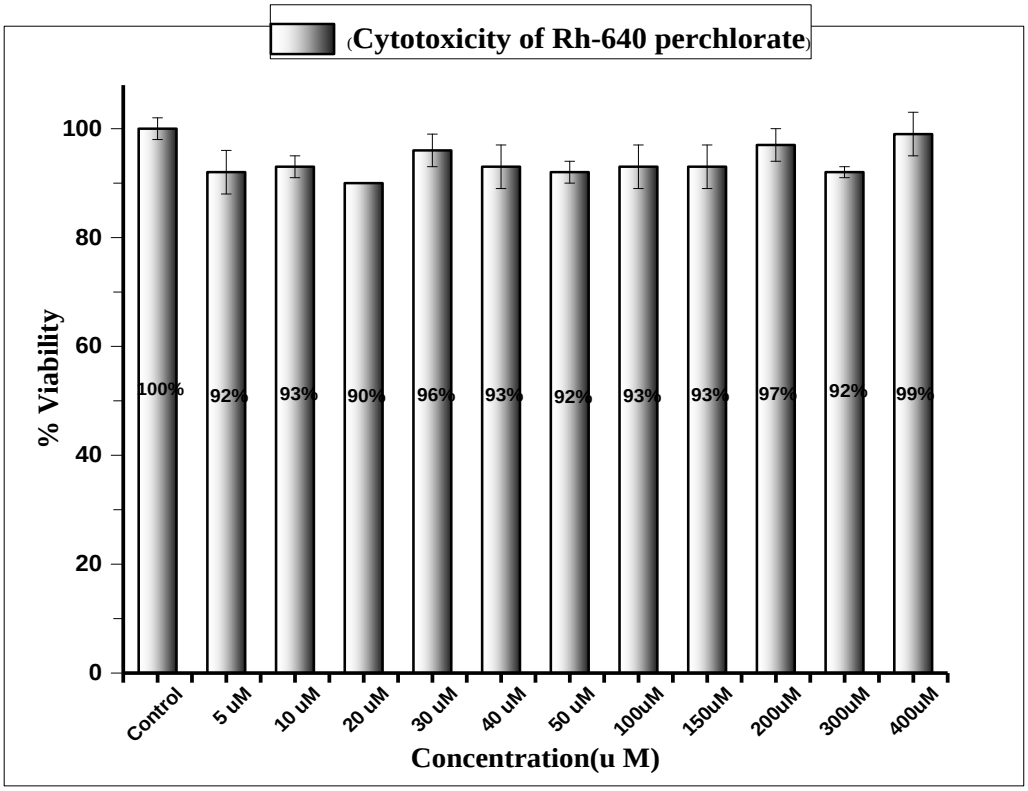

Figure 5: The %viability of RD cells incubated with Rh-640 perchlorate at different (0 – 400 µM) concentrations.

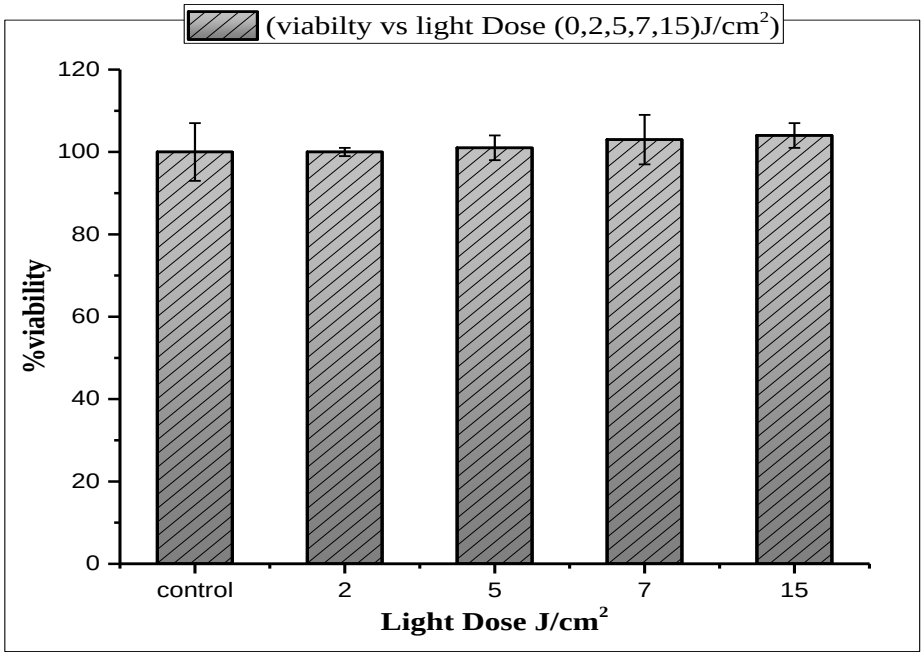

Figure 6: %viability of RD cells non-treated with Rh-640 perchlorate and just treated with laser light (J/cm^2).

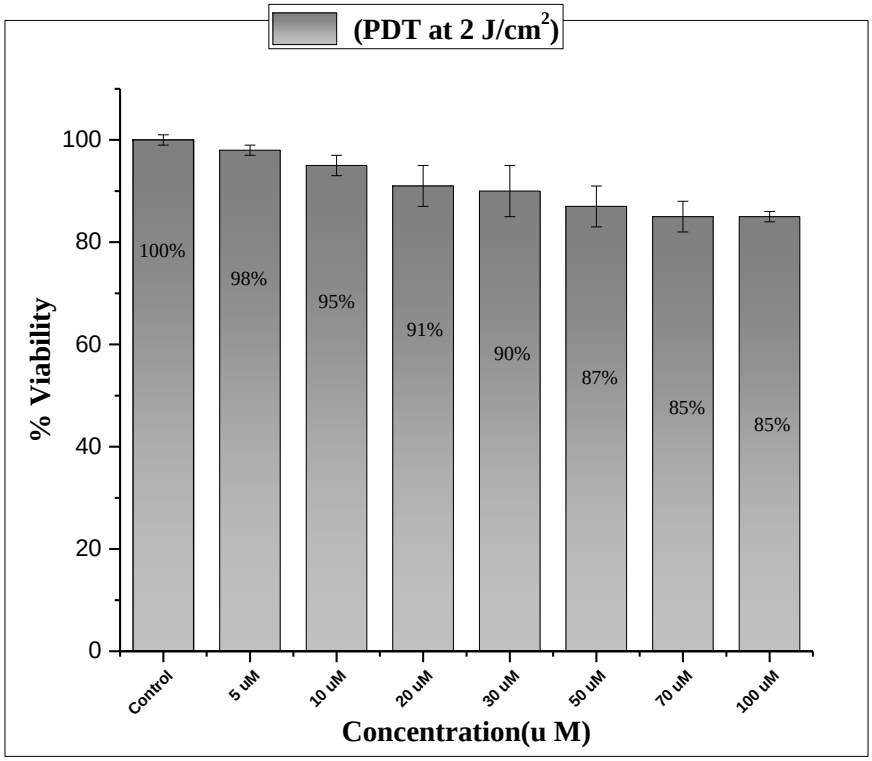

Figure 7: PDT at $2J/cm^2$ at different concentrations (ranging from 0-100 µM) of Rh 640 perchlorate.

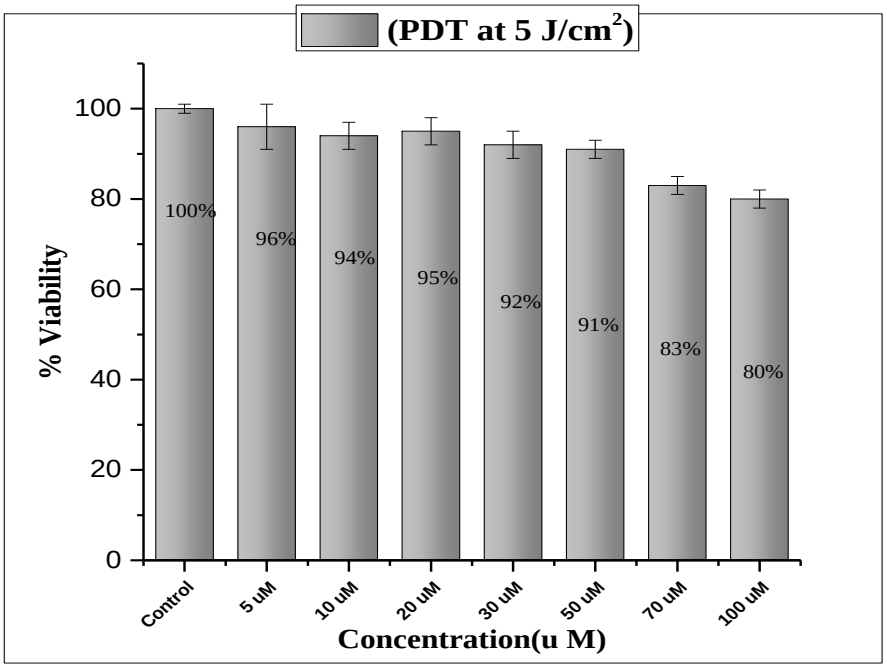

Figure 8: PDT at 5J/cm^2 at different concentrations (ranging from 0-100 µM) of Rh-640 perchlorate.

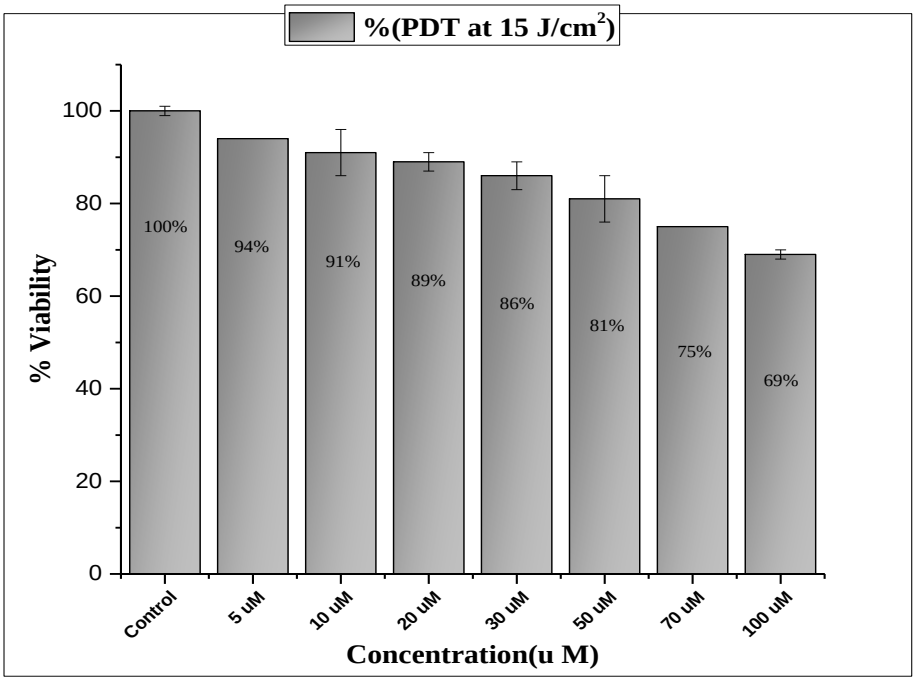

Figure 9: PDT at 15J/cm^2 at different concentrations (ranging from 0-100 µM) of Rh 640 perchlorate

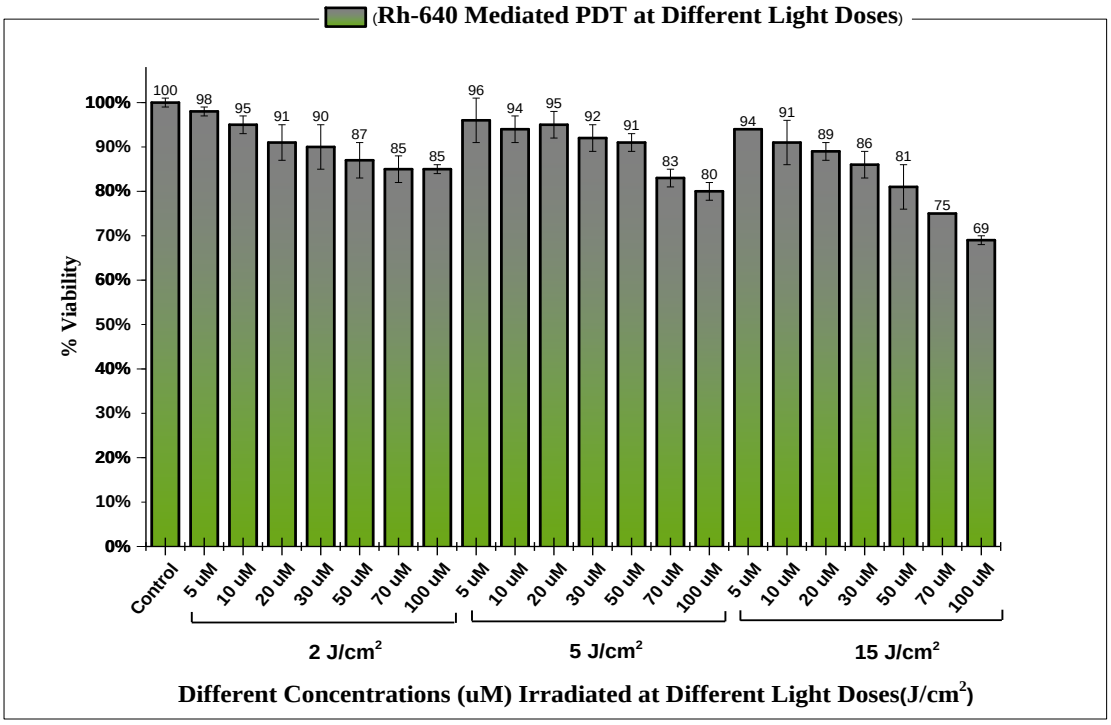

Figure 10: PDT of RD cells incubated with 0-100 µM concentration doses of Rh-640 perchlorate and treated with 2J/cm^2, 5J/cm^2 and 15J/cm^2 of laser light dose (λ = 630 nm).

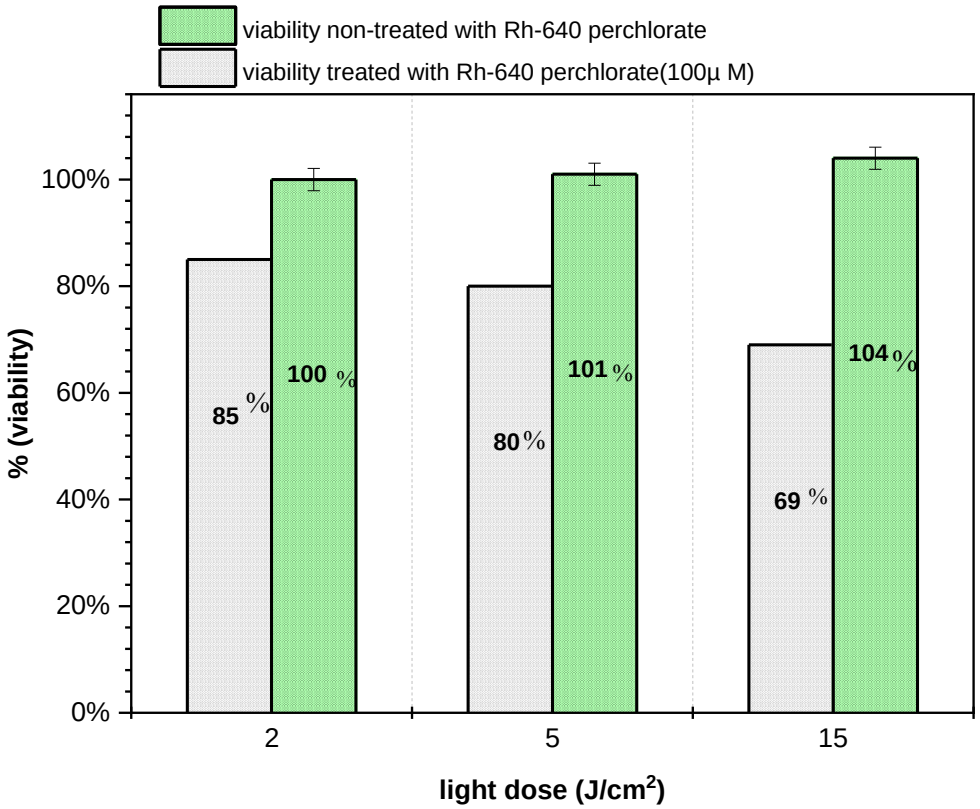

Figure 11: PDT of RD cells incubated with 100 μM concentration and without any dose of Rh-640 perchlorate and treated with 15J/cm^2 of laser light dose (λ = 630 nm).

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