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Original Article

The combination of low level laser therapy and efavirenz drastically reduces HIV infection in TZM-bl cells☆

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ABSTRACT

Background: Human immunodeficiency virus (HIV) infection remains a global health challenge despite the use of antiretroviral therapy, which has led to a significant decline in the mortality rates. Owing to the unavailability of an effective treatment to completely eradicate the virus, researchers continue to explore new methods. Low level laser therapy (LLLT) has been widely used to treat different medical conditions and involves the exposure of cells or tissues to low levels of red and near infrared light. The study aimed to determine the effect of combining two unrelated therapies on HIV infection in TZM-bl cells.
Methods: In the current study, LLLT was combined with efavirenz, an HIV reverse transcriptase inhibitor to establish their impact on HIV infection in TZM-bl cells. Both the HIV infected and uninfected cells were laser irradiated using a wavelength of 640 nm with fluencies of 2–10 J/cm^2.
Results: The impact of HIV, efavirenz and irradiation were determined 24 h post irradiation using biological assays. Luciferase assay results showed that the combination of LLLT and efavirenz significantly reduced HIV infection in cells, despite the undesirable effects observed in the cells as demonstrated by cell morphology, proliferation and cell integrity assay. Flow cytometry results demonstrated that cell death was mainly through necrosis while fluorescence microscopy showed the production of reactive oxygen species (ROS) in HIV infected cells.
Conclusion: Efavirenz and LLLT significantly reduced HIV infection in TZM-bl cells. Furthermore, the death of HIV infected cells was due to necrosis.

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Human immunodeficiency virus (HIV) causes acquired immune deficiency syndrome (AIDS), and was first recognized in the early 1980s when an increased number of young men died after suffering from rare malignancies and unusual opportunistic infections [1,2]. Since its identification, more than 35 million people have died from the infection, with Sub Saharan Africa being the heavily stricken region in the globe [3]. Extensive research has been conducted towards finding treatment for HIV, and antiretroviral drugs are now available and can reduce the virus to undetectable levels. The use of antiretroviral drugs has significantly reduced AIDS related deaths, improved and prolonged the lives of infected individuals [4,5]. The efficacy of antiretroviral drugs is enhanced by the combination of at least three different drugs, commonly known as the highly active antiretroviral therapy (HAART), which has led to a great success as seen by its ability to minimize the likelihood of the virus developing resistance and significantly reducing the viral load [6,7]. Despite the great success that has been achieved by using HAART, a cure for HIV has not yet been found and the infected individuals have to be on a lifetime treatment [8,9]. To ensure that the virus remains suppressed, proper adherence to treatment is essential [10]. The continuous need to take HAART even when the virus is no longer detectable is due to presence of the virus and the ongoing viral replication in compartments and reservoirs, which continue to drive pathogenic disease processes [8].

HIV antiretroviral drugs are categorized into six prominent classes based on the regions of the HIV replication cycle they target and on their molecular mechanisms, which are nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors (INSTIs), fusion inhibitors (FIs) and chemokine receptor antagonists (CCR5 antagonists) [11]. The efavirenz used in this study is a NNRTI, which works by blocking the functioning of the reverse transcriptase enzyme by binding to a distinct site away from the active site known as the NNRTI pocket [12]. Reverse transcriptase is responsible for the conversion of viral genomic RNA to complementary DNA (cDNA), which is integrated into the host genomic DNA [13].

For decades now, low level laser therapy (LLLT) has been used to treat diverse medical conditions. The first therapeutic effects of laser irradiation were observed after the hair of irradiated mice grew back quicker than in that of non-irradiated mice [14]. Since then, the prospects of using light for therapeutic purposes grew. It also became apparent that unlike the use of high power lasers that have thermal effects, which cause detrimental changes on the tissues they are exposed to, low powers of lasers used in LLLT have healing effects on tissues and cells [15,16]. LLLT is a form of photobiomodulation which can be simply defined as the exposure of cells or tissues to red/near infrared laser with wavelengths between 600 and 1100 nm in order to stimulate cellular functions which leads to beneficial clinical effects when using fluences of 0.4–50 J/cm^2 [2,17]. LLLT has been used for pain relief stimulation of tissue regeneration and for the reduction of swelling and inflammation [18–21]. LLLT has also been successfully used to treat herpes simplex virus-1 (HSV-1) infections [22,23].

The mechanism of LLLT in the treatment of different medical conditions is not fully understood, however it is known that the light gets absorbed by chromophores into the cell mitochondria via the cytochrome c oxidase, which is a primary photo-acceptor for the red and near infrared light range in mammalian cells [17]. The absorption of light stimulates the electron transport chain, which leads to the synthesis of adenosine triphosphate (ATP), production of reactive oxygen species (ROS) and release of nitric oxide (NO) [24–26]. ROS are the free radicals and reactive molecules that originate from molecular oxygen produced during electron transport as by products [27]. It was presumed that these molecules only have damaging effects in cell components, however, recently it has been demonstrated that ROS play significant and beneficial roles in cell signalling processes and other physiological processes [28,29]. The induction of these molecules by LLLT have positive effects as they induce transcription factors like nuclear factor Kappa B (NF-κB) and activator protein-1 (AP-1), which in turn promote gene expression, thereby increasing protein synthesis, cell proliferation, cell migration, production of cytokines and growth factors, as well as increased tissue oxygenation [30–32,15]. Furthermore, the absorption of photons by mammalian cells regulates enzymatic activity and also balances both intracellular and extracellular pH [33]. Yaakobi and colleagues have also reported that LLLT increases antioxidant levels and also play a significant role in protein–protein interactions and establishing proper protein confirmation as it stimulates the expression of heat shock proteins [34].

In our previous study, we observed that LLLT was able to reduce HIV infection in TZM-bl cells and in the current study we investigate the use of LLLT in combination with efavirenz, which is known to block the replication of HIV. The aim of this study was to determine how a combination of two unrelated therapies would influence HIV infection *in vitro*.

Materials and methods

Cell lines and maintenance conditions

The two cells lines that were used are TZM-bl cells (ATCC, PTA-5659) and 293T/17 cells (ATCC, CRL, 11268). The 293T/17 cell line was used for the production of HIV-1 pseudovirus that was used to infect TZM-bl cells. Both cell lines were grown, maintained and cultured in a cell growth medium made up of Dulbeco's minimal essential medium (DMEM, Sigma–Aldrich, D5796), 10% fetal bovine serum (FBS, FBS Superior, S 0615), 0.5% L-Glutamine-Penicillin-Streptomycin (Sigma–Aldrich, G6784) at 37 °C, 5% CO_2 and 85% humidity. Since both cell lines were adherent, trypsin–EDTA solution (Sigma–Aldrich, T4049) was used for cell harvesting experiments as it causes the cells to detach from the flask surface and causes the cells to be in suspension so that they could be counted and transferred from one flask to another. This process was mainly used for cell maintenance and also when cells were to be used in experiments.

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Preparation and titration of ZM53 pseudovirus

For the production of HIV-1 ZM53 pseudovirus, 4 μg of an expression plasmid containing the *env* gene of ZM53 and 8 μg of a plasmid carrying the backbone of HIV-1, but deficient of the env (pSG3ΔEnv) were used to co-transfect 293T/17 cells. The Superfect transfection reagent (Qiagen, 310305) was added in the mixture of the two plasmids in order to facilitate the entry of DNA into cells with ease due to the interaction between the cell surface charges and charges on the Superfect reagent. The plasmids were a donation from Prof Maria Papathanasopoulus, Director of the HIV Pathogenesis Research Unit at the University of Witwatersrand, Johannesburg, South Africa. Using the 50% tissue culture infectious dose (TCID50), the suitable virus titre was determined as previously done by Li and colleagues [35].

Experimental groups

There were three groups of TZM-bl cells used: the first group was uninfected TZM-bl cells that were not infected with the pseudovirus, the second group were infected and the third group were infected cells exposed to the drug, efavirenz. The TZM-bl cells (2×10^5) were infected by adding 20 μl of the ZM53 pseudovirus in a 23.5 mm diameter tissue culture dish containing 3 ml growth media. Both the infected and uninfected TZM-bl cells were incubated at 37 °C, 5% CO_2 and 85% humidity for 48 h. After 48 h of incubation, the third group of cells were prepared by adding 20 μg/ml of efavirenz to the infected cells and further incubated for 30 min at 37 °C. Each group of cells had six culture dishes; the first dish was an unirradiated control and the other five were each irradiated at different fluences between 2 and 10 J/cm^2.

Irradiation of TZM-bl cells with laser

Prior to irradiation, the growth medium in the culture dishes was removed and the cells were rinsed twice with 2 ml of Hank's Balanced Salt Solution (HBSS, Gibco, Life Technologies, 14170–088). The HBSS was then replaced with 1 ml of growth medium and cells were irradiated using a 640 nm diode laser (Coherent), with a laser power of 30 mW at the sample area using fluences of 2–10 J/cm^2. [Table 1] shows laser parameters used for irradiation experiments.

The fluence (⌀), power (P) and the irradiated area (A) were the parameters used to determine the duration of cell exposure to laser using the following formula:

Table 1 Laser irradiation parameters.

Laser parameters	
Wavelength (nm)	640
Power Output at the sample (mW)	30
Type of emission	Continuous wave
Illuminated area (cm^2)	4.2
Irradiation times (s)	606, 1263, 1920, 2526, 3183
Fluences (J/cm^2)	2, 4, 6, 8 and 10
Beam profile	Gaussian

$$t = \frac{\varnothing \times A}{P}$$

Through the laser pulse energy in joules (J) and the effective focal spot area in cm^2, we were able to determine different fluences used, using this formula:

$$\text{Fluence} = \frac{J}{cm^2}$$

After laser irradiation of cells using the setup [Fig. 1], the culture dishes were incubated for 24 h at 37 °C in 5% CO_2 and 85% humidity, followed by biological assays.

Cell morphology

The assessment of cell morphology is essential in cell culture experiments, as it is a physical means of determining whether the cells are healthy or unhealthy. In this study, cell morphology was monitored in order to determine the influence of HIV infection, efavirenz and irradiation on the cells. This examination was done using a light microscope (CKX41, Olympus) and images were taken using a digital camera attached to the microscope.

Cell viability assay

The CellTiter-Glo® luminescent cell viability assay (Promega, Anatech, G7573) is a means of measuring cell viability in culture by quantifying adenosine triphosphate (ATP), which is an indicator of metabolically active cells within a given cell population. The addition of the reagent to the cells results in cell lysis, thereby generating a luminescent signal that is proportional to the quantity of viable cells. Equal volumes of cell suspension and reconstituted reagent were added together and mixed to induce cell lysis. The mixture was allowed to stabilize at room temperature for 10 min in the dark. The luminescence was recorded using the GloMax® Discover System (Promega, Anatech) in relative light units (RLUs).

Cell proliferation assay

The CellTiter 96® non-radioactive cell proliferation assay (Anatech, Promega) is a colorimetric assay used to evaluate cell metabolic activity. It makes use of NAD(P)H-dependent cellular oxidoreductase enzymes that reduce the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl) −2,5-diphenytetrazolium bromide, which is yellow in colour to formazan that is purple in colour [36]. The assay was performed in a 96 well plate by mixing 15 μl of the Dye solution with 100 μl of the cell suspension and the mixture was then incubated at 37 °C for 4 h. After incubation, 100 μl of the Solubilization solution was added and the plate was allowed to stand at room temperature in the dark for 1 h. Prior to measuring absorbance by reading the plate at 600 nm using the Glomax® Discover System, the contents were mixed using a multichannel pipette for even distribution of the newly formed substance (formazan).

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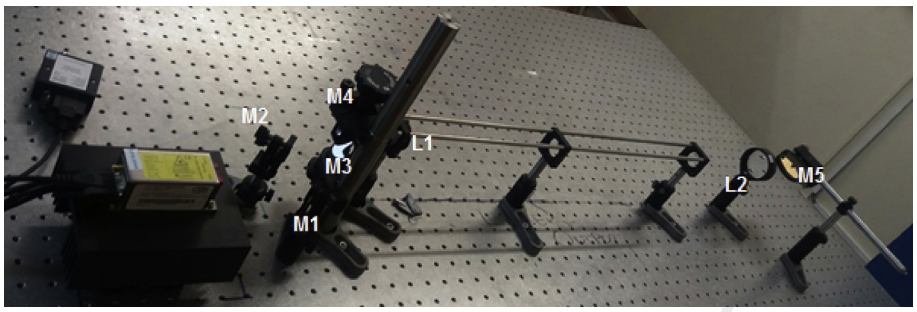

Fig. 1 Low level laser therapy (LLLT) setup in which the beam from a 640 nm laser source was reflected via mirror (M1) to mirror (M2). From M2, the beam was reflected to M3 of the periscope and then transferred to M4. Passing M4 the beam was transmitted by lens (L1) and travelled through a straight optical path to lens (L2), and then transferred to M5 which reflected the beam towards the sample on the optical table where irradiation was performed.

Membrane integrity assay

The CytoTox 96® non-radioactive cytotoxicity assay (G1780, Anatech, Promega) is a colorimetric assay that measures lactate dehydrogenase (LDH) enzyme. LDH is a stable cytosolic enzyme released by damaged tissues and cells into their environment (cell medium). This assay therefore evaluates the presence of tissue and cell damage. It was done by mixing 50 μl of the cell medium with the equal volume of the LDH assay reagent in a 96 well plate and incubating in the dark at room temperature for 30 min. The reaction was terminated by adding 50 μl of the Stop solution and absorbance was measured at 490 nm using the Glomax® Discover System.

Luciferase assay

A luciferase (Luc) reporter gene assay which measures the reduction of HIV as a function of Tat-regulated Luc reporter gene expression after a single round of HIV in TZM-bl cells was done using the Bright-Glo™ luciferase assay system (E2610, Anatech, Promega) to evaluate HIV infection in TZM-bl cells [37]. TZM-bl cells are a CXCR4 positive cell line that expresses both CD4 and CCR5, which are essential cellular surface receptors for the entry of HIV-1 into cells [38]. They also contain integrated Tat-inducible luciferase and E.coli β-galactosidase reporter genes and these two reporter genes get expressed when TZM-bl cells are infected with HIV [39]. Luciferase activity is measured by the RLUs and they are directly proportional to the number of infectious virus particles present in the initial inoculum. For the experiment, 100 μl of the luciferase reagent was added to an equal volume of the cell suspension and incubated in the dark for 2 min. The luciferase activity was quantified with the GloMax® Discover System.

Flow cytometry

The flow cytometry experiments using Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (BD Biosciences, Johannesburg, South Africa) were performed to determine the type of cell death experienced by cells following HIV infection. Following irradiation at different fluences, the cells were incubated for further 24 h, thereafter flow cytometry experiments were conducted. The cell culture medium was removed and placed in a 15 ml centrifuge tube. Following this, cells in the culture dish were rinsed with HBSS, and 500 μl of trypsin was added in each culture dish and placed in a 37 °C incubator for 5 min to allow cells to detach from the culture dish, leaving them in suspension. Once successfully in solution, 1 ml of growth medium was added to the cells and this mixture transferred to a 15 ml tube containing the culture medium and centrifuged for 10 min at 2200 rpm. The cell pellet was then rinsed twice with cold PBS and re-suspended by adding 500 μl of 1X annexin V binding buffer. From the 500 μl cell suspension, 100 μl was stained in 5 ml tubes with 5 μl of annexin-V and 5 μl of propidium iodide (PI) and incubated on ice for 30 min. The stained cells were suspended in 400 μl of annexin V binding buffer before flow cytometric analysis.

Reactive oxygen species detection experiments

Reactive Oxygen Species (ROS) was visualized with CellROX® oxidative stress green reagent (C10444, Life Technologies), which is a DNA dye that binds with DNA upon oxidation and its signal is localized primarily in the nucleus and mitochondria. After irradiation at different fluences, the cells were incubated for 1 h under normal cell growth conditions. After incubation, 750 μl of the growth medium was removed and only 250 μl remained in the culture dish. To the 250 μl medium, 1 μl of 25 mM CellROX green reagent was added and the contents of the dish were mixed by gently swirling to ensure even distribution of the reagent throughout the plate and incubated for 30 min. The cells were washed three times with 1X Phosphate Buffered Saline (PBS) and 500 μl of PBS was left in the dish to prevent desiccation during sample viewing under the fluorescent microscope (CKX41, Olympus).

Statistical analysis

All experiments were conducted in triplicates and repeated three times (n = 3), with average values used to plot the graphs. A two-way analysis of variance (ANOVA) test was used to compare groups, fluence levels as well as interactions

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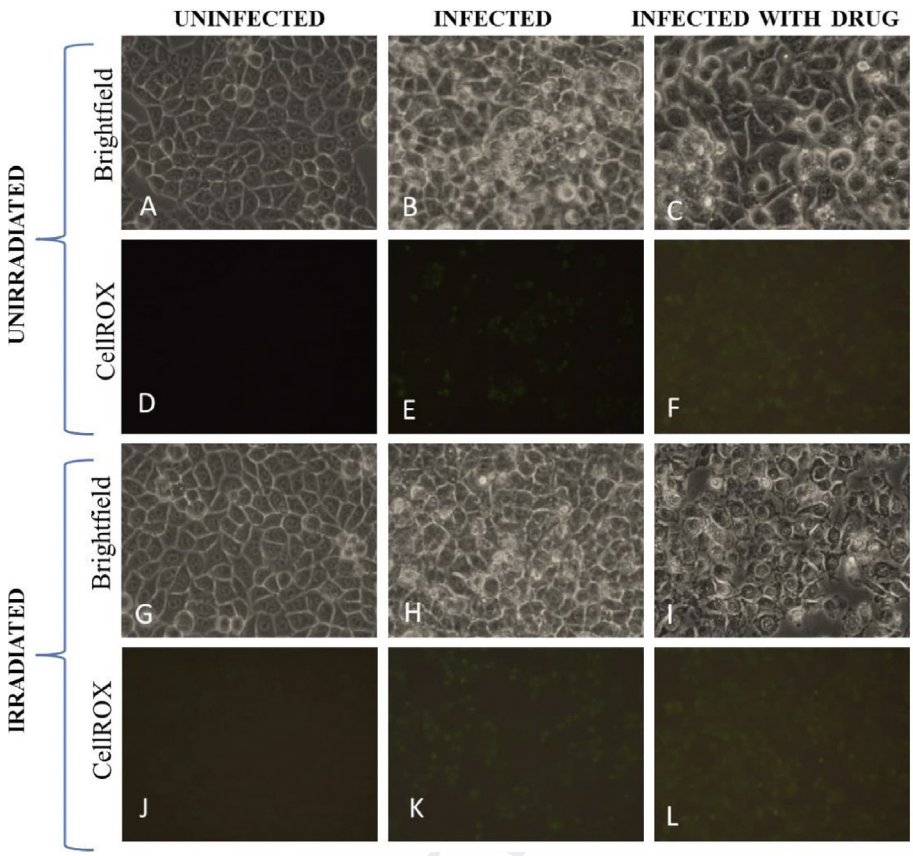

Fig. 2 Cell morphology and ROS production in TZM-bl cells: A to C and G to I is the cell morphology of (A) uninfected unirradiated, (B) infected unirradiated, (C) unirradiated infected with drug, (G) uninfected irradiated, (H) infected irradiated and (I) irradiated infected with drug. D to F and J to L are the ROS production images (D) uninfected unirradiated, (E) infected unirradiated, (F) unirradiated infected with drug, (J) uninfected irradiated, (K) infected irradiated and (L) irradiated infected with drug.

between groups and fluences. The results were considered significantly different at $P < 0.05$ and less. All the non-irradiated cells were used as controls; with uninfected cells being the negative control (NC), infected cells being the positive control (PC) and the infected cells with drug was the drug control (DC).

Results

Effects of infection, irradiation and efavirenz on TZM-bl cell morphology and ROS production

The results in [Fig. 2] show how HIV-1 infection, irradiation and efavirenz in TZM-bl cells had an impact on both the cell morphology and ROS production. The assessment of cell morphology using the bright-field microscope showed that both irradiated and unirradiated uninfected cells in [Fig. 2A and G] were healthy and grew as a monolayer. Also, there was no ROS production in both irradiated and unirradiated uninfected cells as exhibited by the absence of green fluorescence in [Fig. 2D and J].

Both the irradiated and unirradiated infected cells in [Fig. 2B and H] showed signs of cell stress as they became round with some floating around. A similar pattern of stressed cells was observed in both irradiated and unirradiated infected cells with drug [Fig. 2C and I]. With regards to ROS production in infected cells, both irradiated and unirrradiated in [Fig. 2E and K] showed ROS production. ROS production also took place in both irradiated and unirradiated infected cells with drug as demonstrated in [Fig. 2F and L].

Effects of infection, irradiation and efavirenz on cell viability

Cell viability was assessed using adenosine triphosphate (ATP) assay in order to determine the effects of HIV infection, efavirenz and laser irradiation [Fig. 3]. The results showed that

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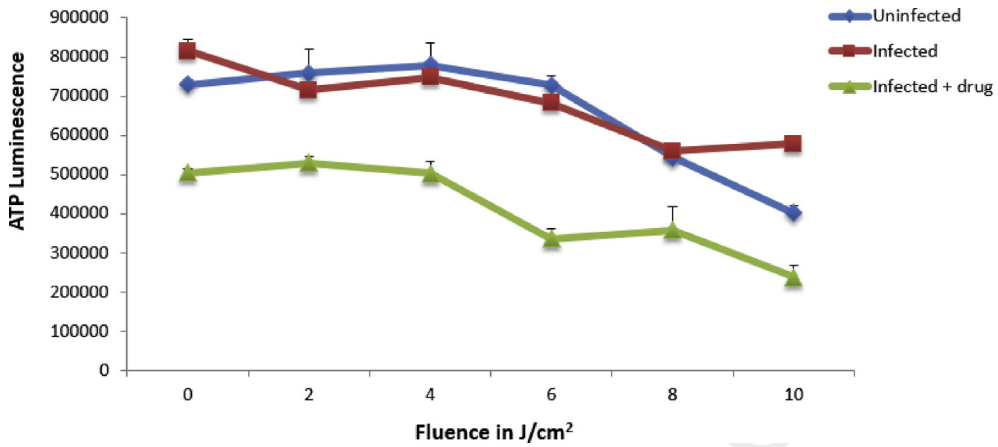

Fig. 3 Cell viability of uninfected, HIV infected and HIV infected TZM-bl cells with efavirenz. Cells were irradiated with increasing fluences of 0, 2, 4, 6, 8 and 10 J/cm^2. Significant differences between groups, fluences and interaction effect were $P < 0.0001$. Error bars represent the standard error of the mean where n = 3.

infected cells treated with efavirenz had the lowest ATP levels compared to the uninfected and the infected cells. According to the two-way ANOVA test, there was a significant difference between groups ($P < 0.0001$) and between fluence levels ($P < 0.0001$) as well as a significant interaction effect ($P = 0.0001$).

Effects of infection, irradiation and efavirenz on cell proliferation

Cell proliferation was assessed using the MTT assay in order to determine the effects of HIV infection, efavirenz and laser irradiation on the proliferation of TZM-bl cells [Fig. 4]. The results showed that both groups of infected cells proliferated poorly in comparison to the uninfected cells. According to the two-way ANOVA test, there was a significant difference between the groups ($P < 0.0001$) and between fluence levels ($P < 0.0001$), however, there was no significant interaction effect ($P = 0.9153$).

Effects of infection, irradiation and efavirenz on cell membrane integrity

The cell membrane integrity was examined using lactate dehydrogenase (LDH) assay in order to determine the effects of HIV infection, efavirenz and laser irradiation [Fig. 5]. LDH is a cytosolic enzyme that is released from cells when the membranes of the cells are damaged. The results showed that the uninfected cells released the lowest levels of LDH into the medium compared to the two groups of infected cells. The infected cells treated with efavirenz released the highest LDH levels. According to the two-way ANOVA test, there was a significant difference between the groups ($P < 0.0001$) and between fluence levels ($P < 0.0001$), as well as a significant interaction effect ($P = 0.0401$).

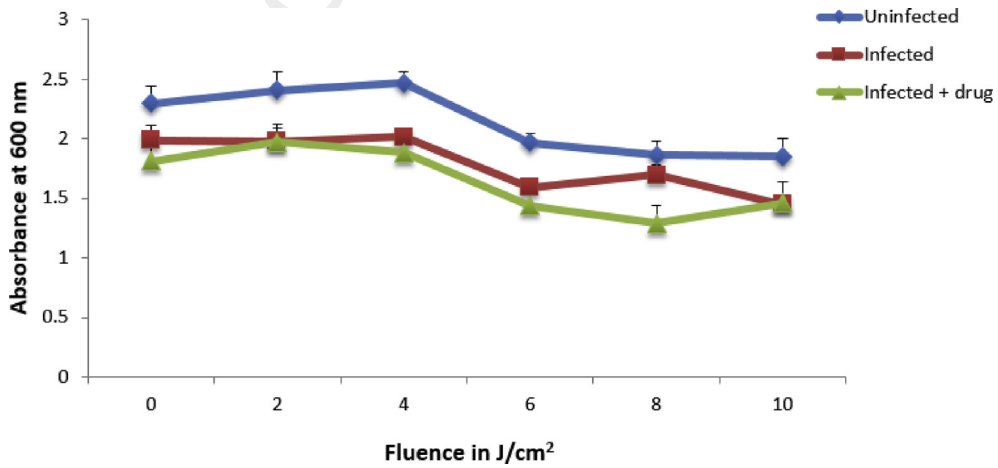

Fig. 4 Cell proliferation of uninfected, HIV infected and HIV infected TZM-bl cells with efavirenz. Cells were irradiated with increasing fluences of 0, 2, 4, 6, 8 and 10 J/cm^2. Significant differences between groups and fluences were $P < 0.0001$ and interaction effect was $P = 0.9153$. Error bars represent the standard error of the mean where n = 3.

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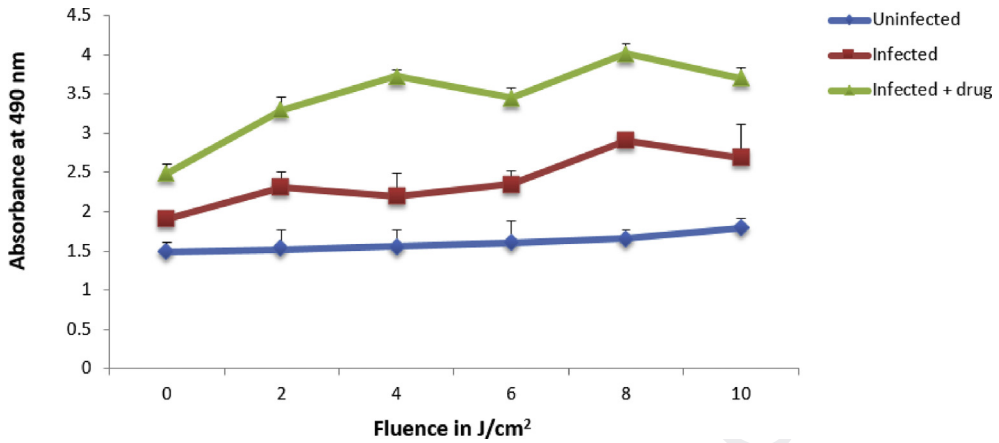

Fig. 5 Cell membrane damage of uninfected, HIV infected and HIV infected TZM-bl cells with efavirenz. Cells were irradiated with increasing fluences of 0, 2, 4, 6, 8 and 10 J/cm^2. Significant differences between groups and fluences were $P < 0.0001$ and interaction effect was $P = 0.0401$. Error bars represent the standard error of the mean where $n = 3$.

Effects of irradiation and efavirenz on HIV infection

The luciferase assay was used to monitor HIV infection and to evaluate the effects of efavirenz and laser irradiation on HIV infection [Fig. 6]. As expected, no HIV infection was detected in uninfected cells and the infected cell treated with efavirenz exhibited lower HIV infection levels when compared to the infected cells. According to the two-way ANOVA test, there was a significant difference between groups ($P < 0.0001$) and between fluence levels ($P < 0.0001$), as well as a significant interaction effect ($P < 0.0001$).

Infection, irradiation and efavirenz cause cell death by either necrosis or apoptosis

The Annevin V-FITC apoptosis kit was used to distinguish between apoptotic and necrotic cells using flow cytometry. [Fig. 7] shows flow cytometric dot plots differentiating between live, necrotic, late apoptotic (dead) and early apoptotic cells and their percentages are shown in [Table 2]. Early apoptotic cells were positively stained with annexin V-FITC (lower right quadrant), necrotic cells were positively stained with PI (upper left quadrant), dead cells were stained with both annexin V-FITC and PI (upper right quadrant) while live cells are not stained (lower left quadrant). Uninfected cells irradiated at 2–10 J/cm^2 showed no changes in cell population when compared to the uninfected control cells and the majority of the cell population was live cells. Infected cells irradiated with 2–10 J/cm^2 also showed no changes in cell population when compared to infected control cells, except for cells irradiated with 8 J/cm^2. These cells had a significantly reduced proportion of viable cells 1.3% ($P < 0.05$) and a significant increase in dead cells 86.6% ($P < 0.001$) and necrotic cells 11.7% ($P < 0.001$).

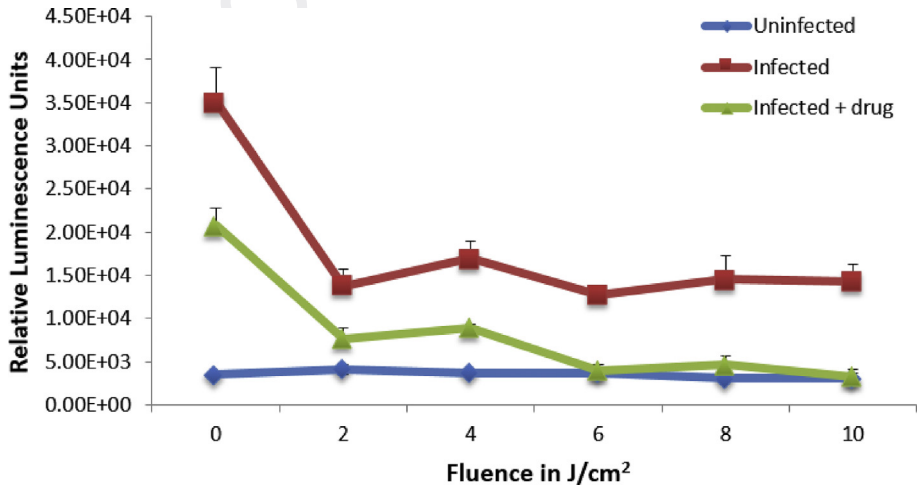

Fig. 6 Luciferase assay monitored HIV infection in TZM-bl cells. TZM-bl cells were irradiated at 2–10 J/cm^2. Significant differences between groups, fluences and interaction effect were $P < 0.0001$. Error bars represent the standard error of the mean where $n = 3$.

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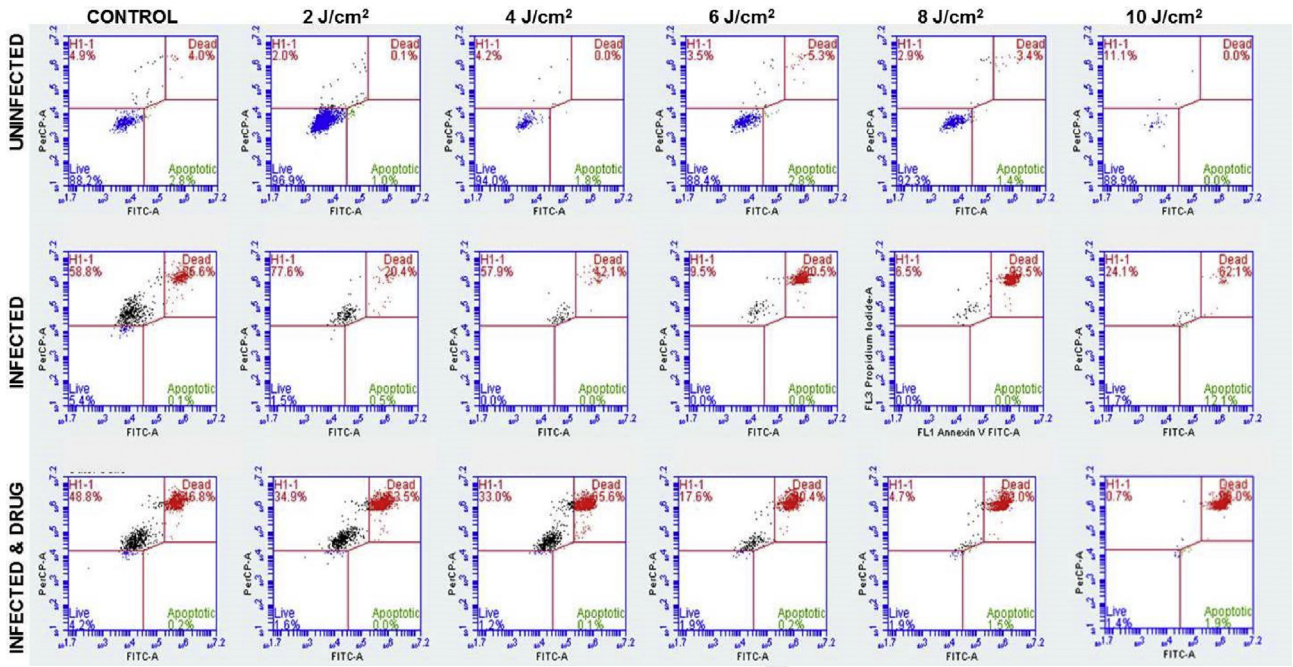

Fig. 7 Propidium iodide/Annexin V FITC staining used to assess the mode of cell death in both irradiated and non-irradiated cells.

Table 2 Percentage of various cell populations after staining with propidium iodide/annexin v-fitc.

Groups	Lives cells (%)	Dead cells (%)	Apoptotic cells (%)	Necrotic cells (%)
Uninfected	93.6 ± 2.7	1.37 ± 1.3	1.27 ± 0.81	3.7 ± 0.84
2	95 ± 0.9	1.1 ± 0.5	0.7 ± 0.35	3.17 ± 0.84
4	92.2 ± 2	2.26 ± 1.39	1.4 ± 0.23	4.1 ± 1.07
6	92.3 ± 2.01	2.46 ± 1.46	1.83 ± 0.61	3.33 ± 0.38
8	93.5 ± 0.63	1.83 ± 0.99	1.23 ± 0.12	3.36 ± 0.73
10	92.3 ± 1.6	1.6 ± 0.8	1.06 ± 0.5	5.16 ± 2.96
Infected	6.43 ± 1.29	38.33 ± 1.56	0.43 ± 2.72	54.96 ± 2.72
2	4.53 ± 1.71	28.73 ± 4.29	0.36 ± 0.08	66.33 ± 5.91
4	2.3 ± 1.21	46.13 ± 3.13	0.3 ± 0.15	51.23 ± 3.89
6	2.3 ± 1.38	73.37 ± 8.58	0.03 ± 0.03	24.2 ± 7.46*
8	1.3 ± 0.68*	86.8 ± 3.66***	0.2 ± 0.1	11.7 ± 3.09***
10	1.9 ± 0.11	58.46 ± 6.91	0.46 ± 0.36	35.53 ± 8.74
Infected and drug	3.67 ± 0.29	37.2 ± 5.48	0.2 ± 0.11	58.97 ± 5.65
2	2.2 ± 0.34*	55.56 ± 4.08	0.4 ± 0.31	41.83 ± 3.51
4	3.53 ± 1.52	56.87 ± 8.48	0.33 ± 0.28	39.17 ± 6.83
6	1.83 ± 0.06*	74.83 ± 4.10**	0.37 ± 0.12	22.97 ± 4.18
8	3.1 ± 1.00	78.33 ± 7.97*	1.37 ± 0.35	17.2 ± 7.27*
10	1.97 ± 0.42*	87.9 ± 4.07**	0.9 ± 0.55	9.23 4.27**

Significant differences between controls and their respective control groups are represented on the graph as (**) = $P < 0.01$ and (***) = $P < 0.001$. Experiments were repeated 3 times (n = 3).

Infected cells treated with efavirenz only showed a reduction in the live cell population when irradiated with 2, 6, and 8 J/cm^2 and a significant increase in dead cells at 6, 8 and 10 J/cm^2 while a reduction in necrotic cell population was only noted at 8 and 10 J/cm^2.

Discussion

The introduction of antiretroviral agents has resulted in millions of lives being spared from the unmerciful scourge of HIV

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infection on the human race. Nonetheless, there is still a dire need to find a form of therapy that would completely eradicate HIV and minimize the side effects caused by the combination of different drugs in each HAART regimen. The efavirenz used in the current study is a first generation NNRTI that has been in use as a first line regimen [40,41]. Since its first use in the late 1990s, efavirenz is still part of the treatment used by HIV infected individuals resulting in viral suppression even after years of using it and this is a clear indication that it is very effective against HIV [41]. In this study, we tested a combination of two unrelated therapies (LLLT and efavirenz) against HIV infection in TZM-bl cells. LLLT has been successfully used in the treatment of various medical conditions such as herpes simplex virus 1 (HSV-1) infection without any side effects. Since LLLT can be effectively used to treat viral infections, its potential in controlling HIV-1 infection was explored in this study [42].

TZM-bl cells are susceptible to HIV primary isolates and molecularly cloned Env-pseudotyped viruses, like the ZM53 virus used in the current study. When HIV infection has taken place in TZM-bl cells, the reporter gene expression is induced by the HIV Tat protein, as the reporter genes are tat-inducible. The presence of reporter genes in TZM-bl cells enables the user to determine whether there is HIV infection or not. Both the negative control and all the irradiated uninfected cells showed no luciferase activity, which was expected as luciferase activity in TZM-bl cells is only induced when HIV infection has taken place [43]. The little luminescence detected in uninfected cells could be attributed to the autoluminescence which is present at very low levels in most mammalian cells [44]. The notably high levels of luciferase activity as shown by RLUs in the presence of HIV was a clear indication that TZM-bl cells are permissive to HIV infection and the ZM53 virus used was infectious. An observation similar to the one made in a previous [45], showed that irradiation caused reduction in HIV infection as seen in [Fig. 6]. Since LLLT promotes processes such as gene expression and protein synthesis, thereby strengthening the immune system, it can be hypothesized that the reduction of HIV levels is due to the stimulation of the immune system rather than inhibitory effects on the virus [30–32,42]. However, at this stage the elaborate scientific explanation and mechanisms on how laser irradiation reduced HIV infection is still unclear. As predictable, was further reduced in infected cells treated with efavirenz and laser irradiation as shown in [Fig. 6]. Even though the reduction of HIV infection was normal in the presence of an antiretroviral agent, the reduction of the infection when the two therapies were combined was a rejuvenating factor, as it is apparent that the combination of the two therapies has potential in fighting HIV infection.

Having assessed how irradiation and efavirenz affected HIV infection in TZM-bl cells, it was essential to establish how the two therapies affect TZM-bl cells. This was done by evaluating cell morphology (light microscope assessment), cell viability (ATP assay), cell proliferation (MTT assay) and cell membrane damage (LDH assay). In [Fig. 2A, B, C, G, H and I], cell morphology results showed visible differences between infected and uninfected cells, and the presence of round cells in the infected cell groups was a clear evidence of what is known about HIV, that it has cytopathic effects on virus permissive cells [46]. The cell morphology results correlate with both the MTT and LDH assay results in [Fig. 4] and [Fig. 5] as the uninfected cells proliferated well and released low LDH levels when compared to the other two groups. Unhealthy and dying cells release LDH in their environment and cease to multiply and this was observed in both groups of infected cells.

With the understanding that HIV infection causes cytopathic effects in cells, one would have expected that HIV infected cells would lower ATP levels in comparison to the uninfected cells. However, the results in [Fig. 3] exhibited some similarities in ATP levels between infected and uninfected cells. A similar observation was seen in other studies where there were high ATP levels in HIV infected cells. The rise of ATP level in HIV infected cells was attributed to the role of the HIV-1 p2 peptide, which intensifies the virus' acute infection and thus increasing the production of intracellular ATP [47,48]. The reduction in cell viability levels in the presence of efavirenz is in correlation with what is known about the detrimental effects of efavirenz in cells [49]. Furthermore, this observation tallies with what was observed in LDH and MTT assay results as they showed the most undesirable effects in cells that were treated with efavirenz.

Flow cytometry was done to determine the mode of cell death associated with HIV infection, LLLT and efavirenz. The flow cytometry results of uninfected cells in [Fig. 7] showed expected results as there was a high number of live cells (88–96%) compared to apoptotic, necrotic and dead cells that had low populations ranging from 0.1% to 11%. The presence of dead or dying cells in a healthy sample was also not an alarming observation as cells die when they are old and are replaced by new ones or when they have been damaged [50]. The dying of old cells in order to be replaced by new ones is a programmed form of cell death that is commonly known as apoptosis. In addition, since TZM-bl cells are adherent, during passaging they get exposed to trypsin–EDTA so as to enable them to detach from the culture flask and a small population of cells gets damaged during this process, thereby leading to cell death. Unlike uninfected cells that had a high population of live cells, the infected cells had a small population of live cells ranging from 0 to 5.4%, while necrotic and dead populations had the highest numbers and the apoptotic population had no greater than 13%. This observation correlates with the other assays where HIV infection, efavirenz and laser irradiation at some fluences had detrimental effects on TZM-bl cells. It also became apparent that cell death in this study was mainly due to necrosis, which correlates with literature where necrosis has been mostly observed *in vitro*, while apoptosis seems to be the most common form of cell death observed *in vivo* [51]. Furthermore, cell death by necrosis is also in correlation with the fact that necrosis occur as a consequence of infection or injury.

There was no ROS detected in both irradiated and unirradiated uninfected cells, which is similar to what was previously observed by Basso, where there were no detectable ROS production in healthy irradiated cells [52]. This result also correlates with what was observed in other biological assays conducted in this study. On the other hand, our results showed what was speculated in the late 1980s, that HIV infection is associated with ROS as cells infected with HIV

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(both irradiated and unirradiated) showed ROS production [53]. Unirradiated infected cells with or without efavirenz showed the same fluorescence pattern with irradiated cells indicating production of ROS. According to these findings, it is clear that the ROS production was caused by HIV infection.

Conclusion

The outcomes of the study showed that LLLT at fluences of 2, 4, 6, 8 and 10 J/cm^2 significantly reduces HIV infection in TZM-bl cells. Furthermore, the combination of LLLT and efavirenz causes a further reduction in the HIV infection. This shows that the combination of the two therapies is powerful in fighting HIV infection and might yield good clinical outcomes. HIV infection in the presence or absence of efavirenz induces cytopathic effects and ROS production in TZM-bl cells. The prevailing mechanism of cell death in the HIV infected cells is necrosis. Further studies to understand the mechanism/s involved in the reduction of HIV infection by LLLT are required. The success of this study would result in the development of portable devices that would be used by HIV infected individuals in both private and public spaces; such as homes and hospitals. The prospective device is also intended to be used with ease even in resource limited settings as it would use batteries instead of power.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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