Medical Journal of Babylon
Vol. 13- No. 1: 7 -16 , 2016
http://www.medicaljb.com
ISSN 2312-6760©2016 University of Babylon

Original Research Article

The Effect of Diode Laser 635nm on Mitochondrial Membrane Potential and Apoptosis Induction of CHO47cells line

Rana Ayad Ghaleb
College of Medicine, University of Babylon, Hilla, IRAQ

E-mail: rana.a.ghaleb@gmail.com

Accepted 21 December, 2015

Abstract

Several studies suggest that low power laser irradiation is capable of affecting cellular processes under different conditions.In this study, the effect of low-power laser irradiation on CHO47 cells was investigated.The cells were irradiated with a CW, 635 nm Diode laser at an energy density ranging from 2.94 to 41.16 J/cm2 (power output, 100mW). The cells were grouped into: group1 control (not irradiated); group 2 exposed to 2.94 J/ cm² ; group 3 exposed to 20.58J/ cm²; and group 4 exposed to 41.16J/cm2. The mitochondrial membrane potential (MMP) of the irradiated cellsand apoptosis assaywas assessed at 96, 120 and 144 hours.In comparison with the control populations, a significant increase in mitochondrial membrane potential (MMP) of irradiated cells was observed. In addition, the irradiated cells showed a significant decrease in early, late apoptotic and dead cells in comparison with their respective controls.Based on the conditions of this study, we concluded that the low-level laser inhibit the apoptotic process and increase mitochondrial membrane potential (MMP).

Key words:CHO cells, Laser irradiation, Apoptosis, mitochondrial membranepotential

الخلاصة

اقترحت دراسات متعددة ان اشعة الليزر ذو القدرة الواطئة لها قابلية التاثير على العمليات الخلوية تحت ظروف متعددة. تم ذلك بدراسة تاثير اشعة الليزر ذو القدرة الواطئة على خلايا مبيض الهامستر. عرضت الخلايا الى ليزر الدايود وبطول موجي ٥٣٦ نانوميتر في نمط مستمر وبكثافات طاقة مختلفة تتراوح من ٢.٦٩-٤١.١٦ جول/ سم^{٢}(وقدرة١٠٠ ملي واط). قسمت الخلايا الى المجموعة الاولى سيطرة (غير مشععه) المجموعة الثانية تعرضت إلى شعاع الليزر بكثافة طاقه ٢.٦٩ جول/سم^{٢}والمجموعه الثالثه تعرضت الى شعاع الليزر بكثافة طاقه ٢٠.٥٨ جول/سم^{٢}والمجموعةالرابعةتعرضت لشعاع الليزر بكثافة طاقه ٤١.١٦جول/سم^{٢}. تم قياس جهد غشاء المايتوكوندريا للخلايا المشععه و وعملية موت الخلايا بعد ٩٦,١٢٠,١٤٤ ساعه. بالمقارنة مع خلايا السيطرة لوحظت زياده معنويه في جهد غشاء المايتوكوندريا للخلايا المشععه.بالإضافة الى ذلك لوحظ نقصان معنوي للخلايا المشععه في طور موت الخلايا المبكر والمتاخر مقارنه مع خلايا السيطرة. اعتمادا على هذه الظروف نستنتج ان الليزر ذو القدرة الواطئة يثبط عملية الموت ويزيد جهد غشاء المايتوكوندريا.

Introduction

Mammalian cells are commonly used for production of recombinant protein drugs and therapeutics in the biotechnology industry. Growthof mammalian cells usually needs an optimizing conduction. Some of cells prefers adhesion onto a surface while some of cells require to suspend to provide a substantial amount of growth surface [1] .

The CHO47cell line which was used in this study is a high producing for protein production. This cell line is different form CHO22H11previously used for monoclonal antibody production. It consider as a medium producing cell line.Another approach is to study the effect of low level laser radiation on CHO47cell line at several energy density

(Dose). The medical use of low level laser radiation inbiostimulation has been observed in many medical area [2]. Mitochondrial DNA replication was activated, cell regeneration stimulated and mitochondrial membrane potential (MMP) increased after exposed to low level laser radiation without modification on cell metabolism[3].Low level laser irradiation has non-significant or even an inhibitory effect on cancer cell proliferation through increased apoptosis at low dose[4].In contrast, some investigators have found that laser radiation has destructive and inhibitory action on cells[5]. Cellular behavior against stress of environmental condition is characterized by induction of apoptosis.Apoptosis is a cell death process characterized by biochemical and morphological features such as reduction in cell volume, loss of microvilli and blebbing of cytoplasm[6,7].The mitochondrialelectrical potential ($\Delta\Psi m$) exerts a control role in the regulation of majorcellular functions, such as calcium signaling, permeability of mitochondrial pores and apoptosis[8]. Mitochondriaplays a central role in cellular homeostasis and it measurements can be used as an early indirect indicator for apoptosis.The main function of mitochondria is toproduce ATP, the energy form of the cells by converting the food molecules by combining oxygen. Themitochondria are the center for the metabolism and apoptotic signaling pathway in cells[9].In the present study, the cell line has an optimal environmental culture condition to grow as a monolayer such as initial cell density, PH and temperature is also found to have effect on the growth rate[10].

The aim of this research was firstly to evaluate the effects of non-agitated environmental condition and low-level laser irradiation (LLLI) on apoptosis mechanism of CHO47 cell linebased on the annexin-V affinity assay for detecting viable, necrotic and apoptotic cells in CHO47 cell line by using flow cytometry.Secondly was to measure the mitochondrial membrane potential (MMP)using flow cytometry.

Materials and Methods

Cell Culture

CHO47 from CHO-K1SV cells waspurchased by LONZA Biologics(Slough, UK). Itgrows in single cell suspension culture and in chemically defined, animal component-free media in a different size of tissue culture flasks. In this research study we changed the culture of CHO47 cell line on plastic tissue culture plates (T-flask 25cm^2) to study the effect of area space on apoptosis mechanism where the space becomes limiting .

Experimental Design of Laser Irradiation

After plating, the CHO47 cells we exposed to irradiations using the diode laser at a wavelength of 635 nm, power of 100mW, and continuous wave. The irradiation with 635nm laser light was made according toAl-Rubeaiand Fernandes[5,11] at the doses of (2.94 , 20.58 and 41.16) J/ cm^2 in term of deposit energy, representing a low, medium, and high doses. The irradiated area was 16 mm^2.

The plated cells were divided into four groups: group 1 as a control (not irradiated); group 2 (2.94 J/cm^2); group 3 (20.58 J/cm2); and group 4 (41.16 J/cm^2). Irradiation of the cells was carried out immediately after plating**.**According to Medrado[12],all wells were coveredin order to avoid any accidental influence.

Mitochondrial Potential Assay

Measurements of normalized mitochondrial membrane potential were performed as described byPande[13]. This assay was performed using two mitochondria staining dyes. These two dyes are a reduced MitoTracker Red CM-H2Xros and a mitochondrial membrane potential sensitive fluorescence (mmp) that quantifies mitochondrial activity. Rhodamine 123 and chloromethyl-X-rosamine

(CMXRos)canbeused as a probe to measure the mitochondria transmembrane potential (TMP) using flow cytometry[14].Mitotracker green can be used to provide an indicator on the mitochondrial content. A combination ofboth fluorochrome can give a ratio of cells that are active (normalized mitochondrial activity)[15].Cells at concentration $5X10^5$ cells/ml were taken from culture suspension. The Red CM-H2Xros dye working concentration was 200nM. Then 0.5 µl of MitoTracker Red CM-H2Xros dye was added to 500 µl of cells sample. The stained cells were incubated at 37ºC in dark for 30 minutes. Sample were run on the Beckman Coulter Quanta SC flow cytometry. Negative control sample (non-stained cells) were also used in this study. The data obtained from FC were analyzed using (FCS Express 4 Flow Research Edition) software to get the MMP according toChow,Klaunig and Isenberg[16,17].

Apoptosis Measurement Using Annexin-V-FITC Assay

Cells of 10^6were washed with PBS and centrifuged at 200g for 5 min. The cell pellet was re-suspended in 100µl Annexin-V-FITCbinding buffer (2µg/ml) final concentration of Annexin-V-FITC. The cells are then suspended in binding buffer where 1µl of Annexin-V was added to 100µl of cell suspension and incubated for 10 minutes at room temperature. After the incubation period 400µl of binding buffer were added to the sample. 10µl ofPropidium Iodide (PI) was added to the appropriate tubes and an incubation of 1 minute was performed[18]. The samples were analyzed with Beckman Coulter Quanta SC flow cytometer with a diode laser at 488nm and a 525nm band pass detection filter to obtain FITC fluorescence.While the maximal emission of PI fluorescence was collected at 670nm long pass.

Results

Mitochondrial Membrane Potential Analysis Using FC

The flowcytometric results showed that mitochondrial membrane potentialincreased significantly ($P<0.01$) at doses of 2.94, 20.58 and 41.16 J/cm^2 respectively in comparison to the control group on day 5. A significant increase was observed in mitochondrial membrane potential ($p<0.01$) at dose of 2.94J/cm^2 on day 4 and 6 (Fig.1). The data obtained from FC were analyzed using (FCS Express 4 Flow Research Edition) software to get the MMP as shown in Fig. 2.

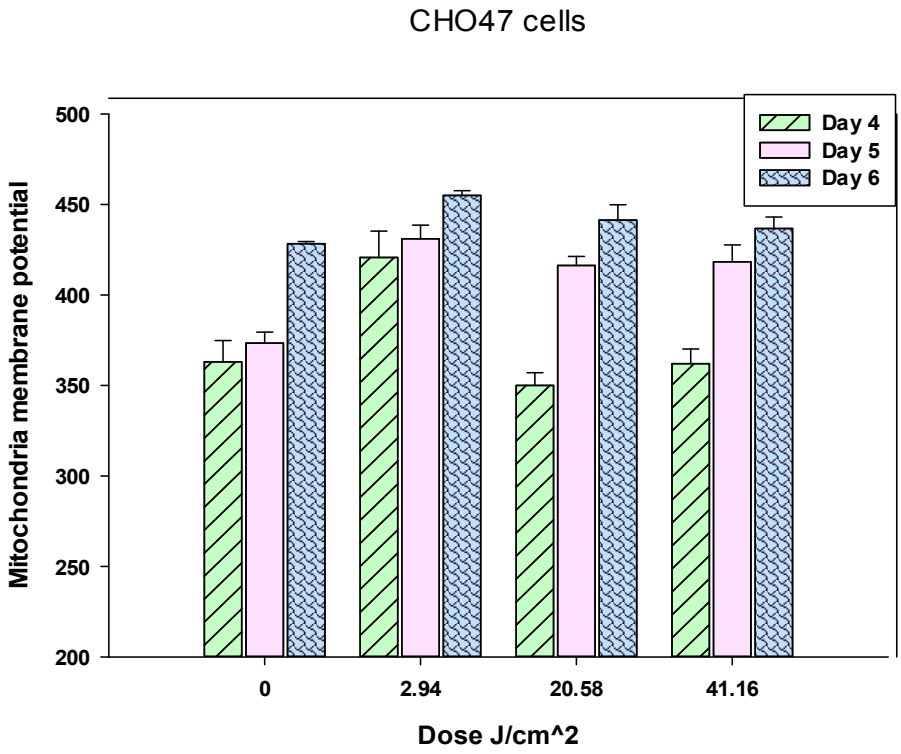


Figure 1: Mitochondrial membrane potential of CHO47 after the exposure to various laser doses

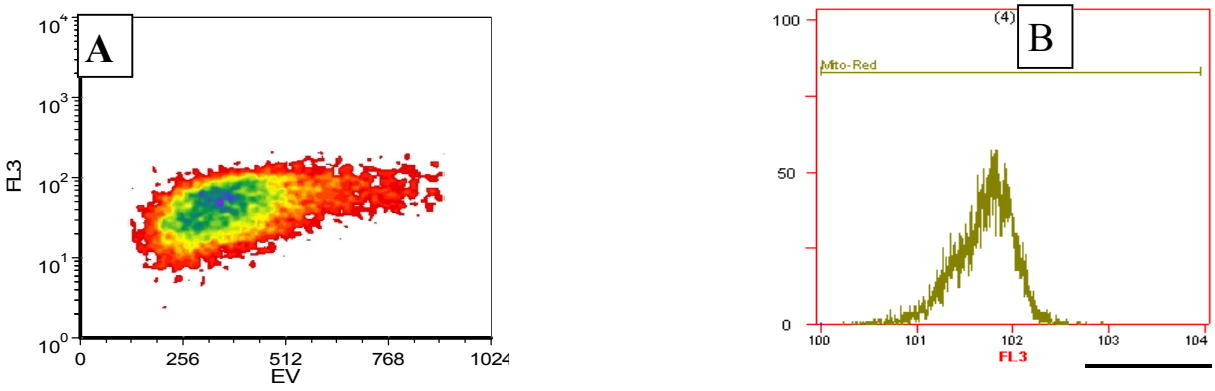
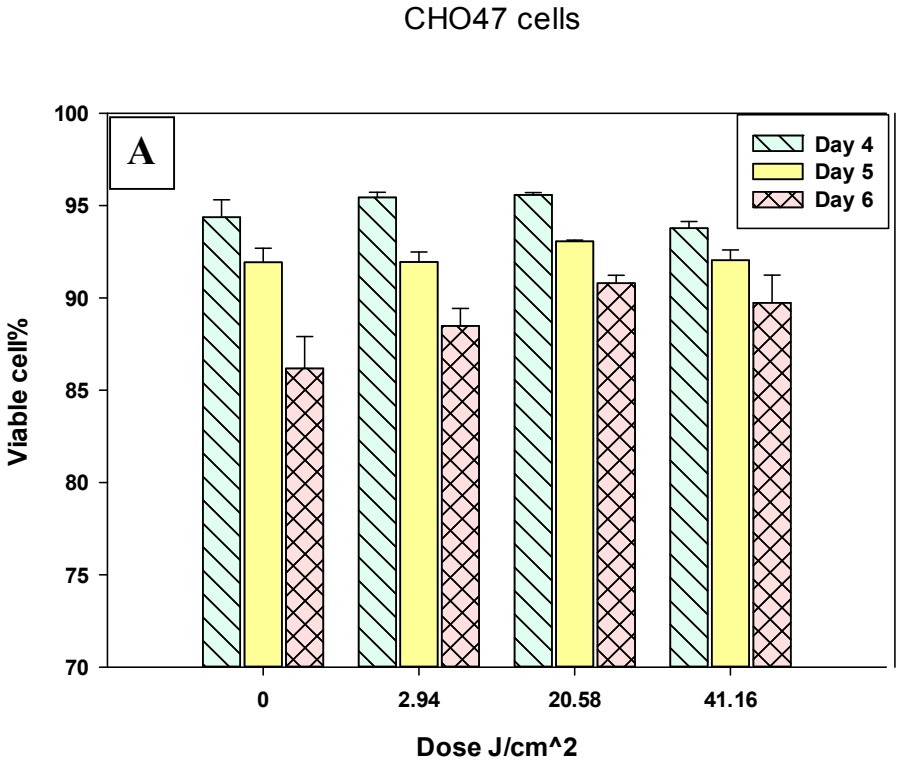


Figure 2: Mitochondrial membranepotential of CHO47 (A) software analysis result (B) FC result

ApoptosisAnalysis Using Flowcytometry

The FC results showed that viable cells fraction have not significantly increased at the doses of (2.94, 20.58 and 41.16) J/ cm² in comparison to the control group. A significant increase ($p<0.01$) in early apoptotic cells percent was observed at the dose of 41.16 J/cm^2 on day 4 and 5. For the dose of 20.49 J/cm^2 there was a significant decreases ($p<0.001$) in the early apoptotic cells percent on day 6. A significant decrease ($p<0.01$) in late apoptotic cells percent was observed at the doses of (2.94 J/cm^2 on day 4 and 6), (20.49 J/cm^2 on day 4, 5 and 6) and 41.16 J/ cm² on day 6. A significant decrease ($p<0.01$) in apoptotic (dead) cells percent was observed at the doses of 2.94 J/cm^2 and 20.49 J/cm^2 on day 6 as shown in Fig.1 and Fig.2.



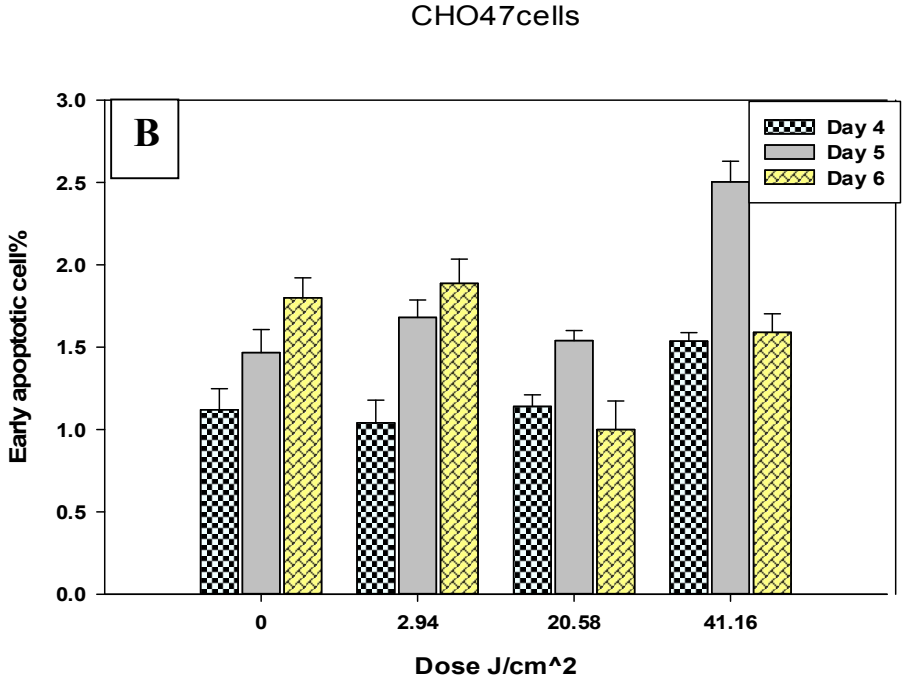
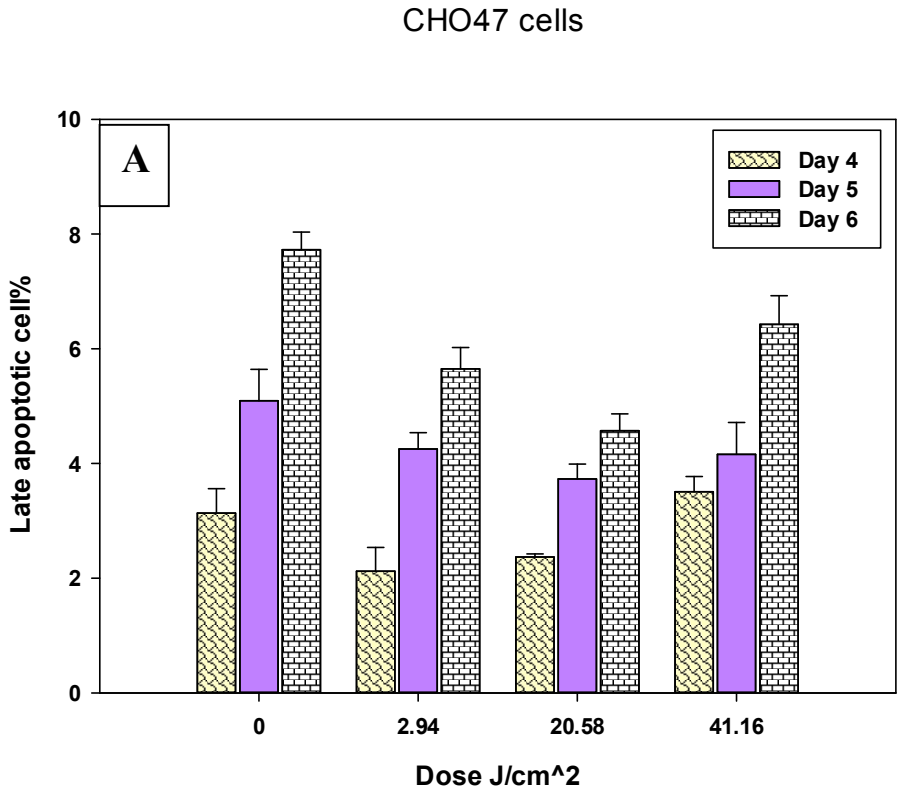


Figure 1:Effect of various laser doses of Chinese hamster ovary (CHO47) cells on apoptosis mechanisms (A) % viable (B) % early apoptotic %



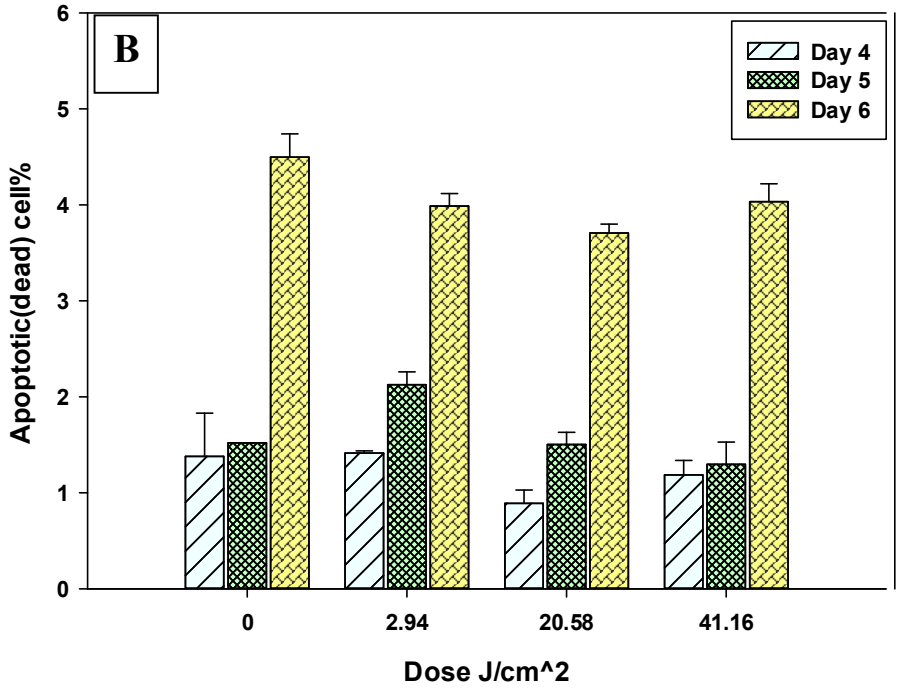


Figure 2:Effect of various laser doses of Chinese hamster ovary cell (CHO47) cells on apoptosis mechanisms (A) % late apoptotic (B) % dead

Discussion

The photobiological effect of low level laser light on cells and tissues depends on the cell type and wavelength of light source. At low radiation dose photoreceptors propagate cellular responses will be activated. The light will be absorbed by endogenous chromophores such as porphyrins and cytochromes [19].He-Ne and Ga-Al-As lasers (LLLI) were used to stimulate mitochondrial membrane potential (MMP)[20].

The irradiated cells were found to have a higher mitochondrial activity than non-irradiated cells. This may be due to enhance cells size which provides a higher respiratory demand at cellular level translated into higher activity of mitochondria. This results wereagree with previous findings that found a positive relation between cell size and mitochondrial activity[21].Another possible effect is that laser light is absorbed intracellularly by chromophores on mitochondria or by other intracellular organelles or even specific molecules which lead to prevent the apoptosis and reduce the effects of reactive oxygen species(ROS)andoxidative stress[22,23].Intracellular ROS have been regarded as a critical factor in different types of cell death. Among intracellular ROS, $O2^-$ leads to a change in mitochondrial membrane permeability, an initiator of apoptotic progression. Another possible effect may be due to increase in the synthesis of adenosine triphosphate after exposed to low level laserirradiation[24]which stimulates the electron transport chainand increase of the respiratory cell metabolism. ATP production increased also as a result of the proton-motive force (pmf) which leads to release more calcium into the cytoplasm from the mitochondria thus the pmf increases

ATP production[25]. Also the activation of the PI3K/Akt pathways, which controls several intracellular signaling pathways, such as regulation of gene expression[26].

Also this results were corroborate the conclusions ofCarnevalli et al., who found that low level laser promotes positive biomodulation as a prevention factor to reduce cell apoptosis, even under non optimal conditions of cells culture[27-30]. Authors thought that there is a dose-dependent relationship in the inhibition or induction of LLL induced cell apoptosis. The reduction in cell apoptosis were observed in the present research. This may be due to inhibitsstaurosporine (STS)-induced cell apoptosis by inactivating the GSK-3b/Bax pathway after exposed to different laser doses.Low level laser inhibit the activation of GSK-3b, Bax, and caspase-3 induced by STS. Bax as a member of Bcl-2 family, is regulators in the apoptosis of mitochondrial pathway. During the apoptotic stimuli, it translocate from the cytosol to the mitochondria. The Bax translocated to mitochondria may be inhibited bylow level laser[31].The proapoptotic proteins Bax, Bak, Bid, and caspases leads to mitochondrial dysfunction, or permeability transition. The permeability transition results in an efflux of cytochrome c from mitochondria, with subsequent activation of caspase-dependent apoptosis.All these processes inhibited by low level laser. Low level laser may leads to change in the proapoptoticBax and antiapoptotic Bcl-2 ratio[32].These finding was agreement withXingresults[33]in which decrease pro-apoptotic (i.e., Bax) and increase anti-apoptotic proteins after exposed to low level laser.

Acknowledgements

This project was sponsored by Professor Mohamed Al-Rubeai,School of Chemical and Bioprocess Engineering University College Dublin, Ireland.

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