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Intra Cellular Fluorescence for Monitoring The Physiological Changes and Biostimulation of Yeast Irradiated with Low Power 532nm CW Nd:YAG Laser

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Abstract: The purpose of this study is to investigate the biostimulation effect of 532 nm CW laser on the metabolism of Saccharomyces *cerevisiae* yeast. Cells were irradiated by 532 nm Nd:YAG laser using 0.153 W/cm2 power density at 30, 45, 60,180 and 300 seconds exposure times in their respective orders. Intrafluorescence parameters were measured by detection the autofluorescence intensity, proliferation rate and Imaging the fluorescent mitochondria using confocal laser scanning microscope. The results showed that the 30 and 45 second exposure times seem to have stimulated changes in the cells that led to increase proliferation, viability and mitochondrial activity. Autofluorescence of cells increased after 45 and 60 seconds exposure time. After 300 seconds there seems to be very noticeable decrease in proliferation, viability and autofluorescence. Confocal microscopy images showed that here is a correlation between fluorescence intensity using mitochondrial probes and proliferation rates of cells.

Introduction

Saccharomyces cerevisiae is one of the most important fungi in the history of the world. This yeast is responsible for the production of alcoholic beverages and bread and a source of protein and was used in biotechnology and genetics as a host for the genes of other organisms (Madigan, *et al.* 2006). Low energy laser irradiation of which output power is in the range of mW modulates various biological effects and has been shown to have positive effect on living organisms both in vitro and in vivo. However, the true effect of low energy laser on cell proliferation is sill controversial, because of conflicting reports on the effects of visible laser light on the cells in culture (Antonio, *et al*., 2002).

There are many evidences that the most intracellular autofluorescence is due to endogenous fluorophores present in cells include tryptophan , reduced nicotinamide adenine dinucleotide (NADH,NADPH) in the cytoplasm and mitochondria, which serves as a

coenzyme and a principal electron donor within the cell for both oxidative phosphorylation and glycolisis , flavin adenine dinucleutide (FAD), which exist mostly as cofactors of enzymes involved in redox reactions, and collagen Milessa, 2004; Ramanujam, 2000; Benson, 1979; Vladimir, 2009).

Fluorescence emission of NADH detected in the region of 450 nm after excitation at 366 nm. Fluorescence detected in the region of 540 nm after excitation at 460 nm measures the cellular levels of the flavoproteins lipomide dehydrogenase and electron transfer flavoprotien (John, 2006; Masters, 1989; Tsubota, 1988).

Several studies have been reported the effect of green laser light on different cells Wavelengths of 514 and 532 have been reported to change metabolic activities in rat myocardial cells (Berns, 1972). Karu marginally reported in her works absorption of green band light by fibroblast and small DNA synthesis activation (Karu, 1999). Frequency doubled Nd:YAG laser was used to induce biostimulation of dermal fibroblast (Vinicent, 2005). Stimulation of the chicken fibroblast proliferation was induced using LED of wavelength 570nm (Kassak, 2005).

The aim of this work is to study the biostimulation effect of low power 532 nm Nd:YAG laser on the metabolism of yeast *Saccharomyces cerevisiae* by investigation the viability, proliferation rate, intracellular fluorescence using fluorescence microscope and confocal laser scanning microscope.

Materials and Methods

Yeast cells culture

(*Saccharomyces cerevisiae*) yeast was used in the experiments. The cells were cultivated on potato-dextrose agar. Cells were transferred after growing to 250ml-volumetric flask with 50ml of liquid nutritive medium (2% glucose, 1%yeast extract and 0.02% ammonium sulphate, pH 5.5) , incubated in shaker incubator 150rpm for 24h. The cells were grown in liquid nutritive medium at 28° C to obtain the required number of cells at the logarithmic growth phase.

The cells were separated from the nutritive medium by centrifugation (2000 rpm for 5 min) and washed twice with sterile tap water. The suspension with optical density 0.06 (λ=540nm) in K, Na-phosphate buffer (pH=6.0) was prepared for irradiation (Xiao, 2006).

Laser irradiation experiments

Diode Pumped Solid State (DPSS) Second harmonic generation Nd:YAG Laser(Viasho , china) was used in this study. This system is operated at the continuous wavelength of 532 nm and an adjustable power output from 0 to 150 mW. Power output was kept constantly at 30mW in all irradiation experiments. The correct eyewear was worn during irradiation. All irradiation experiments were done inside the laminar flow hood under aseptic conditions. Cells washed twice and suspended in the medium.

Cells were transformed by pipetting into five groups of 0.5 ml ependorff tubes, each group was exposed to laser light at certain exposure time using 30, 45, 60,180 and 300 seconds in their respective orders. Each group with certain exposure time was subdivided into 12 replicates keeping the same number of replicates in each group without irradiation as a control. During irradiation the laser was placed vertically on the top of the tubes. We have chosen a distance of 10 cm between laser aperture and the target surface in order to obtain a laser spot light corresponding to the area of a 5 mm diameter ependorff tube. The power density was calculated to be 0.153 W/cm^2 (Quickenden and Daniels, 1993).

Prior each irradiation, cells were shacked well to maintain the homogeneity of distributed light between cells. The control groups received no laser light but exposed to the same environment for the same duration of laser irradiated groups. Post irradiation, the irradiated and non-irradiated cells were cultivated at optimum growth condition.

Autofluorescence experiments

After 48 hours of incubation the autofluorescence properties of irradiated and non irradiated cells were measured using Spetra MAX M5 Multi-Mode Microplate Reader (Molecular Devices). Prior autofluorescence measurements the media were removed from wells and replaced with 1X phosphate buffer saline to avoid the fluorescence signals coming from medium.

The software of the Microplate reader system were set on fluorescence mode to measure the emission intensity at 450 nm after excitation with 366 nm (Schneckenburge, 1992).

Proliferation rate experiments

Cells proliferation assay post 48 hours of irradiation was performed using Vybrant MTT Cell proliferation Assay Kit (Invitrogen). The MTT assay involves conversation of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide) to an soluble formazan. The formazan is then solubilized, and the concentration is determined by optical density at 570 nm.

The media were removed from wells and replaced with 100µL of fresh culture medium. 10 µL of the 12 mM MTT stock solution were added to each well, include a negative control of 10µL of the MTT stock solution added to 100µL of medium alone. Microplates were incubated at 37 C for 4 hours, then 100µL of SDS-HCL solution was added to each well and mixed thoroughly using the pipette.

After 14 hours of incubation at 28 C, samples were mixed using pipette. The absorbance values were measured at 570 nm using Spetra MAX M5 Multi-Mode Microplate Reader (Molecular Devices) (Avanteka, etal., 2010).

Viability measurement using Acridin orange and Fluourescence microscopy

Cells viability was measured directly after irradiation, 5µg/ml (v/v) Acridine orange solution were added to the equal volume of cell suspension. The percentage of viability was measured using Optika Fluoresence Microscope. The number of viable cells (Green in color) and the number of dead cells or cells undergo apoptosis (Red or Orange) were calculated with in one hundred cells. The total number of viable cells was calculated for irradiated and control samples (Sandrine, et.al. 2003).

Confocal Laser Scanning Microscopy Experiments

In order to investigate the fine 3D structure, Mitochondrial shape and membrane potential fluorescence, Specific Mitochondrial dyes were used to image yeasts mitochondria using Olympus flowview 1000 confocol laser scanning microscope (Microscopy Unit – Department of Biological Science – Macquarie University-Sydney–Auatralia).

Two Mitochondrial probes were used: Mitotracker green (Absorption λ: 490nm, Emission λ: 516) and MitotraCker red (Absorption λ: 581nm, Emission λ: 644) (Fung and Theriot, 1989).

Results

Viability of yeast cells after Laser Irradiation

Figure 1 represents the image of yeast cells under fluorescence microscope after staining with Acridine orange. Viable cells remains green after staining while cells undergo Apoptosis or died cells appeared orange or red. Figure 2 represents the Differential Interference Contrast (DIC) image of non- stained yeast cells using fluorescence microscope.

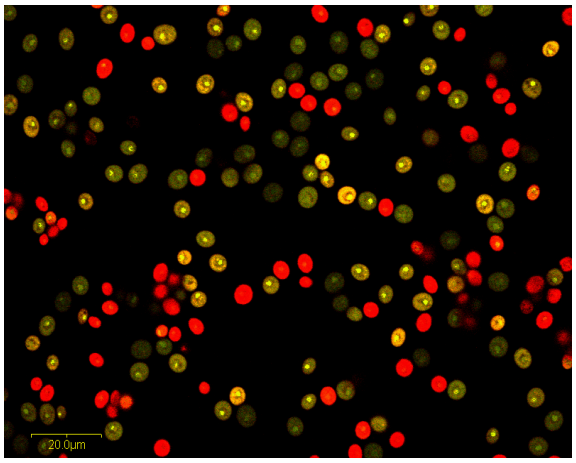

Fig. (1): Fluorescence Image of yeast cells after staining with Acridine orange.

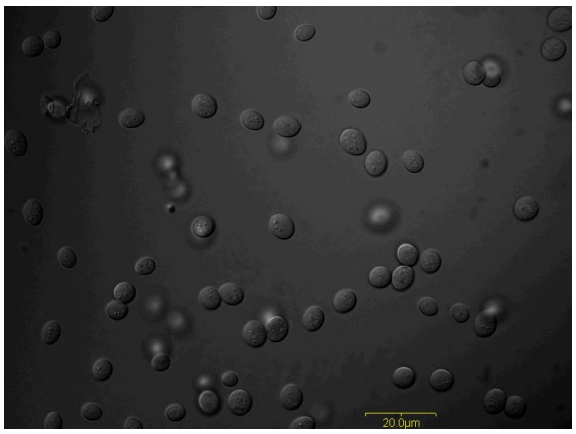

Fig. (2): Differential Interference Contrast (DIC) Image of yeast cells.

The effect of laser light using different exposure times on the viability of yeast cells is illustrated in Figure 3.

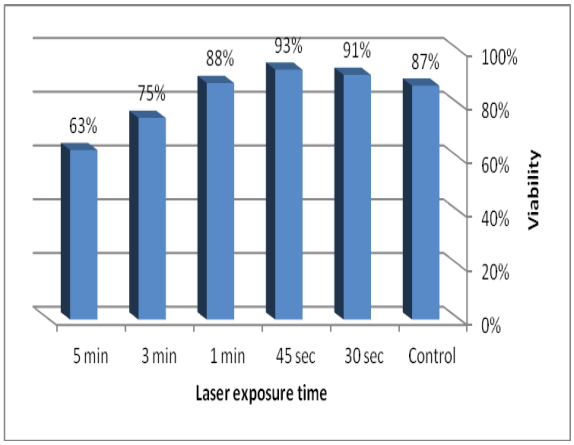

Fig. (3): Viability of yeast cells post irradiation with Nd: YAG laser.

A noticeable increase in the viability of cells can was obtained using 30 and 45 seconds exposure time, while the viability was inhibited using 3 and 5 minutes . No noticeable change in the viability was observed using 1 minute exposure time comparing with control group.

Proliferation rate results

The results of the effect of laser light using different exposure times on the proliferation of yeast cells is illustrated in Figure 4. In this figure, the proliferation is represented by absorbance at 570nm using MTT KIT and micoplate reader. A noticeable increase in the viability of cells can was obtained using 30 and 45 seconds exposure time.

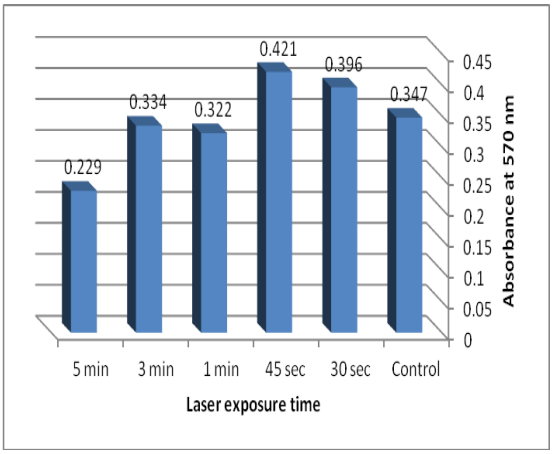

Fig. (4): proliferation of yeast cells in term of absorbance post irradiation by laser.

No noticeable change in the viability was observed using 1, and 3 minutes exposure times comparing with control group while the proliferation was inhibited using 5 minutes.

Autofluorescence results

Figure 5 illustrates The results of the effect of laser light using different exposure times on the intracellular autofluorescence of yeast cells coming from NADH that absorb light at 366 nm and emit the fluorescence at 450 nm.

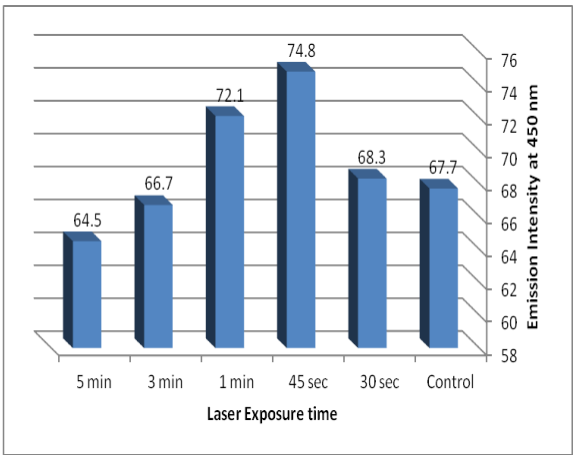

Fig. (5): Intracellular Fluorescence intensity of NADH in yeast after laser irradiation

An increase in the fluorescence intensity can be seen using 45 seconds and 1 minute exposure time, while the intensity is less using 5 minutes. No noticeable change was observed using 30 seconds exposure time comparing with control group.

Confocal Laser Scanning Microscope results

The Confocal Laser scanning microscopy images of yeast cells after irradiation with Nd:YAG laser are illustrated in Figure 6 regarding Mitotracker green and Figure 7 regarding mitotracker red. Changes in the shape, size, budding and mitochondria can be seen in images for both stains.

Cells appeared large in size , high ability of budding (proliferation) and well characterized active mitochondria using 30 and 45 seconds exposure times comparing with control group. Smaller size of cells with less ability for dividing was observed using 1,3 and 5 minutes exposure times.

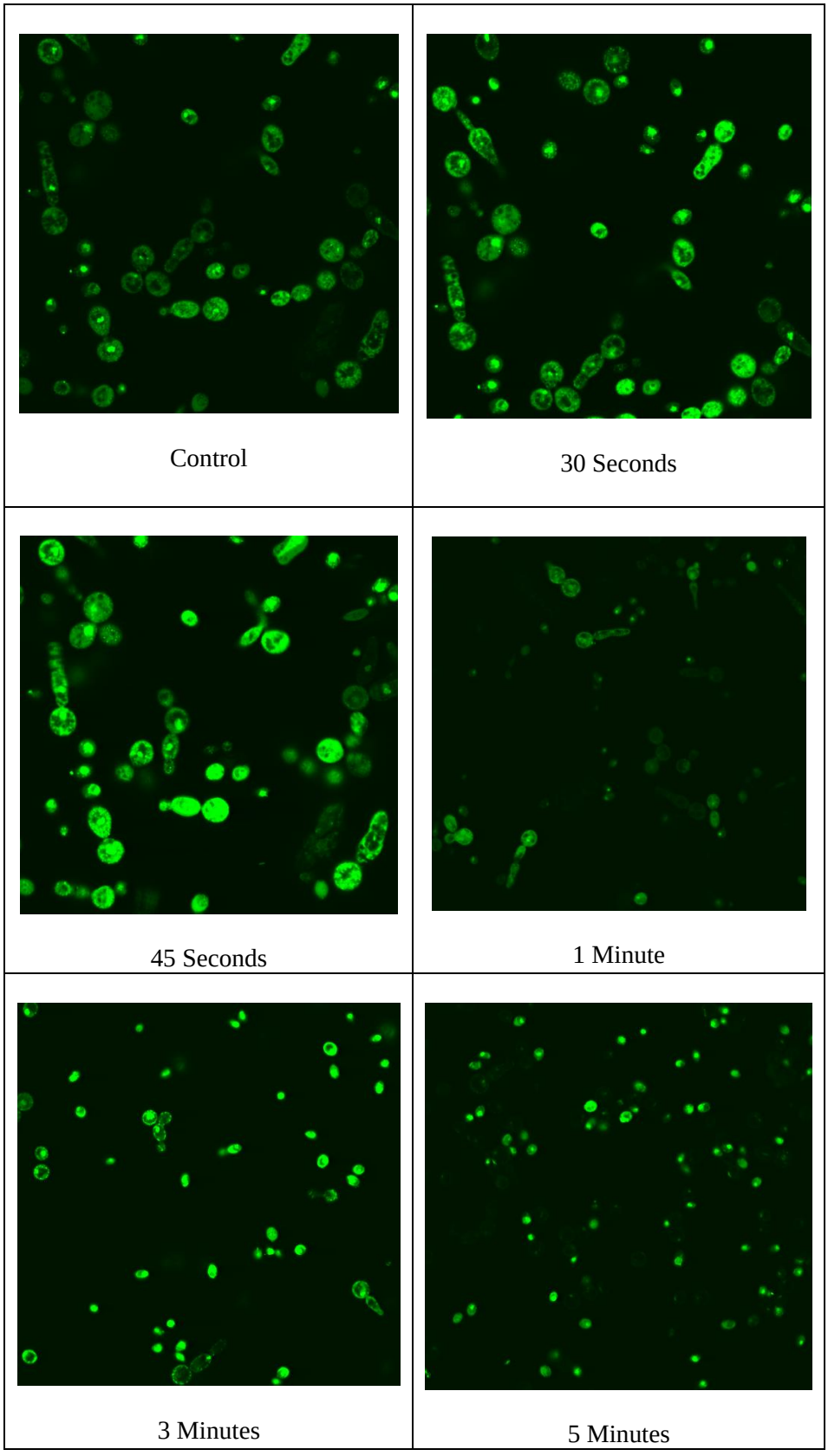

Fig. (6): Confocal laser scanning microscope images of yeast cells using Mitotracker green.

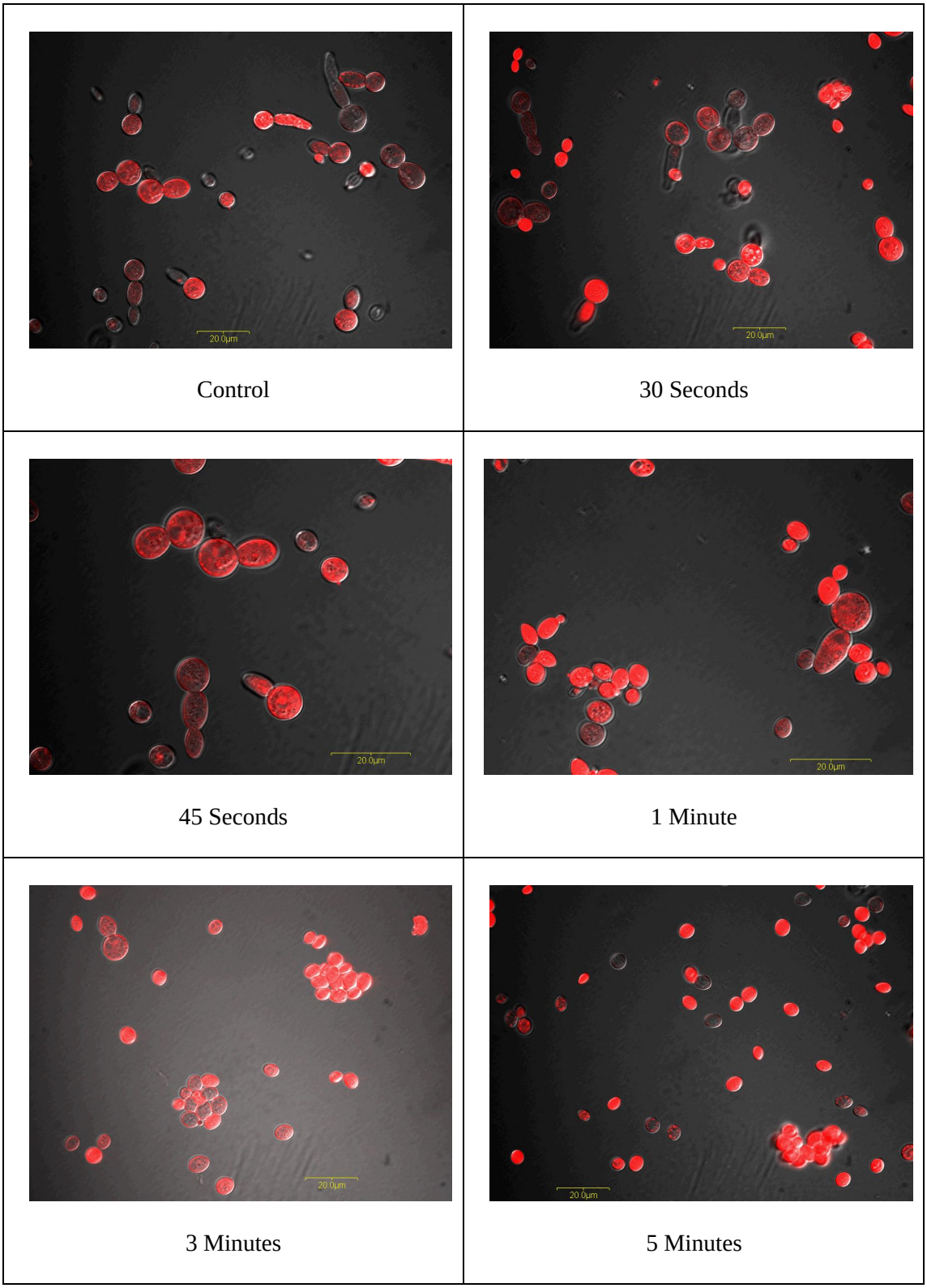

Fig. (7): Confocal laser scanning microscope images of yeast cells using Mitotracker Red.

Discussion and Conclusions

The results of this study showed that 532 nm laser light can increase the proliferation in yeast cells. These findings were supported by measuring the intracellular autofluorescence for an important component of respiratory chain in mitochondria, NADH,taking into account that NADH molecules are involved in the redox reactions of the inner mitochondrial membrane is effected, the states of oxidation of NADH and flavin may changes, this may result in an alteration of the fluorescence intensities (Herbert, et al.,1992) .

A noticeable increase in cells viability was observed after 30 and 45 seconds exposure times, and viable cells appeared in green bright fluorescent color using Acridin orange and fluorescence microscopy, while most of cells appeared dead or undergo apoptosis after 60 seconds, this perhaps due to thermo damaging of cells after long exposure to laser light. The results of autoflourescence showed that the increase in autofluorescence appears at 45 and 60 seconds exposure time and this may be due to increasing in the metabolic activity of mitochondria leading to increase the amounts of fluorescent components of the transport chain in mitochondria.

Staining the cellular mitochondria by fluorescent probes gave an indication about the cellular metabolism after laser irradiation. Confocal microscopy images using two mitochondrial probes, Mitotracker red and green showed good prove about the biostomulation effect of laser in term of increasing the size and number of mitochondria as well the proliferation activity.

Although, it is not known why some cell populations are more highly autofluorescent than others, it seems reasonable that if indeed the fluorescence arises from bound flavins and NADH, the cells' fluorescence intensities reflect the intracellular concentrations of these compounds.

Fluorescence components of reduced pyridine nucleotides in the cytoplasm and mitochondria with greater quantum yield from the mitochondrial bound species.

Different metabolic rates and different cytoplasmic to nuclear volumes could be reflected in larger concentrations of the compounds in one cell than another (Johan, 2006; Aubin, 1979).

The mechanism by which low intensity lasers induce biostimulation of cell activity has been well described by Karu (Karu, 1989). Laser irradiation is postulated to intensify the formation of a transmembrane electromechanical proton gradient in mitochondria (Friedman, et al., 1991). Karu proposed a chain of molecular events starting with the absorption of light by photoacceptor (Chromophore), which leads to signal transduction and amplification, and finally results in photoresponse (Noell, 1976).In this way light is absorbed by components of the respiratory chain, which causes an activation of the respiratory chain and oxidation of the NAD pool, which leads to changes in the redox status of both the mitochondria and the cytoplasm (Karu,1988). This in turn has an effect on membrane permeability/transport, which changes in the Na^+ /H^+ ratio and increases ATPase activity, which in turn has an effect on the Ca^{2+} flux. The Ca^{2+} flux affects the levels cyclic nucleotides, which modulates DNA and RNA synthesis and which modulates cell proliferation (Smith, 1991).

We concluded that there are significant increasing in the auto fluorescence properties, Viability and Mitochondrial activity of yeast cells that have undergone irradiation by 532 nm laser, the 30 and 45 second exposure times seem to have stimulated changes in the cells that led to increase proliferation, viability and mitochondrial activity. An increase in cells autofluorescence occurred at 45 and 60 seconds exposure time. After 300 seconds there seems to be very significant decrease in proliferation / viability and autofluorescence. Confocal microscopy images showed that, there is a correlation shown between fluorescence intensity using mitochondrial probes and proliferation rates of cells

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الفلورة الداخل خلوية لتحديد التغيرات الفزيولوجية و التحفيز البايولوجي للخمائر بعد التشعيع بليزر الندميوم ياك واطيء القدرة بالطول الموجي 532 نانومتر

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الخلاصة

الغرض من الدراسة الحالية هو التحري عن التاثير المحفز لليزر الندميوم ياك بالطول الموجي 532 نانومتر على ايض خلايا الخمائر, تم تشعيع خلايا الخميرة بليزر الندميوم ياك بالطول الموجي 532 نانومتر و بكثافة قدرة 0.153 واط / سم2 و لفترات تشعيع 30 ، 45 ،60 ، 180 ، 300 ، ثانية على التوالي. قيست المعلمات الخاصة بالتفلور الداخلي بواسطة قياس التفلور الداتي ، معدل التضاعف و الفلورة الناتجة عن المايتوكوندريا باستخدام مجهر الليزر الماسح. اظهرت النتائج بان التشعيع للفترات 30 و 45 ثانية يؤدي الى حدوث تحفيز لتضاعف الخلايا والحيوية و فعالية المايتوكوندريا . ازداد تفلور الخلايا بعد فترات التشعيع 45 و 60 ثانية . بعد **300** ثانية من التشعيع ظهر ان هناك انخفاض في تضاعف الخلايا و الحيوية . اظهرت نتائج مجهر الليزر الماسح علاقة بين الفلورة و تضاعف الخلايا باستخدام متحسسات التفلور و معدل تضاعف الخلايا.