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Journal of Photochemistry and Photobiology A: Chemistry 168 (2004) 59–65
Journal of Photochemistry and Photobiology A:Chemistry
www.elsevier.com/locate/jphotochem

Effect of red and near-infrared laser light on adenosine triphosphate (ATP) in the luciferine–luciferase reaction

Albert Amat [a,*], Josepa Rigau [a], Renata Nicolau [a,c], Maurice Aalders [b], Maria Rosa Fenoll [a], Martin van Gemert [b], Josep Tomàs [a]

[a] *Histology and Neurobiology Unit, Faculty of Medicine and Health Sciences, Rovira i Virgili University, C. Sant Llorenç 21, 43201 Reus, Spain*
[b] *Laser Centre, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands*
[c] *Health School, Vale do Paraiba University, Sao Jose Dos Campos, SP, Brazil*

Received 29 April 2004; accepted 25 May 2004
Available online 3 July 2004

Abstract

Adenosine triphosphate (ATP) is an important molecule in biology because it stores chemical energy and releases it to the biochemical processes occurring in the cell. In this study we analysed the biochemical behaviour of ATP after irradiating it with 635 and 830 nm diode lasers. We analysed the luminescence peak, the reaction rate and the area under the luminescence curve at 2×10^{-9} mol/l of ATP in the luciferine–luciferase luminescence reaction before and after irradiating the molecule at several irradiances and radiant exposures. The absorption spectrum of ATP at 3×10^{-3} mol/l concentration was measured between 650 and 900 nm after laser irradiation at 635 nm (Argon-Dye) and 830 nm (diode laser). We found significant differences in the measured parameters when ATP was irradiated with both wavelengths. The absorption spectra of non-irradiated and irradiated ATP show a physical–chemical difference in the ATP molecule after irradiation with both lasers. We can conclude that visible and near-IR laser light with the parameters that were used in this study changed the biochemical behaviour of ATP molecules.

Keywords: Adenosine 5-triphosphate (ATP); 630 and 830 nm diode laser; Firefly luciferine–luciferase photoluminescent reaction; Reaction kinetics; Spectroscopy

1. Introduction

All cells obtain their energy from nutritional compounds (principally glucose) through biochemical reactions that have negative free energy changes (at the end of the process, the free energy of the system decreases because the energy is stored in energetic molecules such as ATP, which are used in most biochemical reactions). Vegetal cells obtain their energy from solar light through photosynthesis. In both of these cases, almost all the free energy is stored in chemical bonds that are to be used in the cell processes. In all organisms, the most important molecule in storing and transferring that energy is adenosine triphosphate (ATP).

Ribonucleoside 5′-trisphosphate acts as a donor of phosphate groups in the cell's energy cycle, transporting chemical energy from one metabolic cycle to another and acting as a shared intermediary that couples endergonic reactions to exergonic reactions. The hydrolysis of each of the high-energy phosphate anhydric bonds of ATP produces a notable change in free energy (−7.3 kcal/mol).

The resulting diphosphate of adenosine (ADP) is recycled (phosphoriled) to ATP by two different mechanisms: chemical energy (during oxidation of nutritional compounds) or light energy (in photosynthetic cells) [1].

One-way laser light is used in medicine by inducing the photochemical effect at low irradiance. A photochemical effect is produced when a chromophore is excited by absorbing a photon and this supplementary energy is transferred for the onset of a biochemical reaction [2]. Karu [3] described the absorption of various wavelengths at the mitochondrial respiratory chain level and defined several unspecific chromophores that change their redox potential to increase the electronic energy levels. This leads to an increase in ATP synthesis. Lubart et al. [4] described a possible intracellular

Abbreviations: ATP, adenosine 5-triphosphate; V_0, peak voltage of the luminescent signal; $V(t)$, exponential function of the luminescent signal; k, constant of the light decay after 1 min; J/cm^2, Joules/cm^2 radiant exposure units

*Corresponding author. Tel.: +34-977-759343; fax: +34-977-759322.
E-mail address: alag@fmcs.urv.es (A. Amat).

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doi:10.1016/j.jphotochem.2004.05.024

signal pathway. Other authors described biochemical and biological responses to irradiation with laser light that are independent of mitochondrial activity and in which no known chromophore is identified [5–10]. However, for all these processes to occur, ATP is needed.

The aim of this study is to analyse the biochemical behaviour of ATP after irradiation with visible and near-IR laser light using the luciferine–luciferase reaction.

2. Materials and methods

Reagents, concentrations and handling: ATP and the firefly luciferine–luciferase complex with Mg^{2+} as cofactor (Sigma FL-AA ATP bioluminescent assay kit). The assay mix is a lyophilised powder containing luciferase, luciferine, $MgSO_4$, DTT, EDTS, bovine serum albumin and tricine buffer salts. It is stable indefinitely if stored desiccated below 0 °C and protected from light. The ATP standard is a pre-weighed lyophilised powder. Each bottle contains approximately 1 mg (2.0×10^{-6} mol) of ATP. It is stable desiccated below 0 °C.

Temperature measurement: The bath temperature of distilled water and ATP samples at all ATP concentrations were monitored during laser irradiation to determine whether laser irradiation produced a change in the temperature of the preparation. During the experiment, the thermistor probe (YSI Reusable Temperature Probe—YSI Incorporated 402; resolution ± 0.2 °C) was placed in contact with the sample and attached to a centralised data logger (Letica—TMP 812).

Light sources: Two AsGaAl diode lasers were used, one at 635 nm with 16 mW output power, and one at 830 nm with 32 mW output power. The spot area in both lasers was 0.25 cm^2. For the absorption spectrum experiments, we excited ATP by light from an Argon-Dye laser tuned at 635 nm and set for 80 mW output power and 12 J/cm^2 radiant exposure, and at 830 nm by a diode laser with the same parameters.

The power was measured with a Melles Griot 13 PEM 001 power/energy meter.

Luminescence detection: We used a LKB Wallac 1250 luminometer measuring at 562 nm and LKB Bromma 2210 two-channel recorder to obtain the graph of the luminescence signal. With this system, we could measure luminescence from small ATP concentrations in the order of attomol/l.

Spectroscopy to measure ATP absorption: We used a Jasco FP-750 spectrofluorometer and varied the wavelengths from 650 to 900 nm in 1 nm steps.

Technical procedure: The assay solution was the assay mix diluted in 5 ml of sterile aqua distillate at pH 7.8 and stored at 0 °C. We made three different ATP solutions, 2×10^{-9}, 2×10^{-10} and 2×10^{-11} mol/l and stored them at 0 °C for 1 h before use. The five control groups were as follows. First, the assay solution alone; second, the three ATP solutions alone; third, fourth and fifth, the assay solution plus each of the three ATP solutions. The ATP samples were made by mixing 100 μl of non-irradiated or irradiated ATP (at different radiant exposures) with 100 μl of non-irradiated assay solution. The ATP samples were heated to 37 °C and then irradiated at 1, 4 and 6 J/cm^2 radiant exposure.

The light emission of the reaction measured by the luminometer began immediately after all the reagents were inserted. A peak in the intensity, expressed as peak voltage V_0 of the luminometer, occurred about 1 s later. As the reaction progressed and the ATP content in the sample decreased, the luminometer voltage, $V(t)$, decayed from the V_0 peak voltage to zero. For the analysis we assumed an exponential decay of the luminescence signal:

$$V(t) = V_0 \exp(-kt)$$

where k is the rate constant of the light decay (in 1/min) and t the time (in min) after the initial peak V_0. We measured $V(t)$ at $t = 1$ min and derived k from:

$$k = \ln\left[\frac{V_0}{V(1\,\text{min})}\right]$$

To assess the absorption spectra, we used ATP samples that were either irradiated or non-irradiated. The area under the luminescence curve was determined by the integral of the measured $V(t)$ curve and is equal to V_0 divided by k (the unit is V min).

Statistical study and data analysis: We took 314 recordings: one each for the first and second control groups, 48 for the third control group and 44 for each of the six irradiated groups (635 and 830 nm at the three different radiant exposures). Statistical analyses were made with GraphPad Prism software, using the ANOVA test complemented with the Bonferroni test. The level of significance was 95% ($P < 0.05$). The data from absorption spectra were processed with Excel software.

3. Results

3.1. Temperature measurement

Temperature recordings were taken for 5 min periods. There were no significant differences between the sample temperatures of the control preparation and the laser-irradiated preparations with the different parameters between 1 and 5 min after irradiation. The room temperature during these experiments was 25.9 ± 0.1 °C.

3.2. Exponential behaviour of V(t)

Six graphs were measured and an exponential decay was observed in all of them. We therefore assumed that all the others were also exponential (see Fig. 1).

Controls: In the first and second control groups, we found no luminescence emission. The third control group gave the

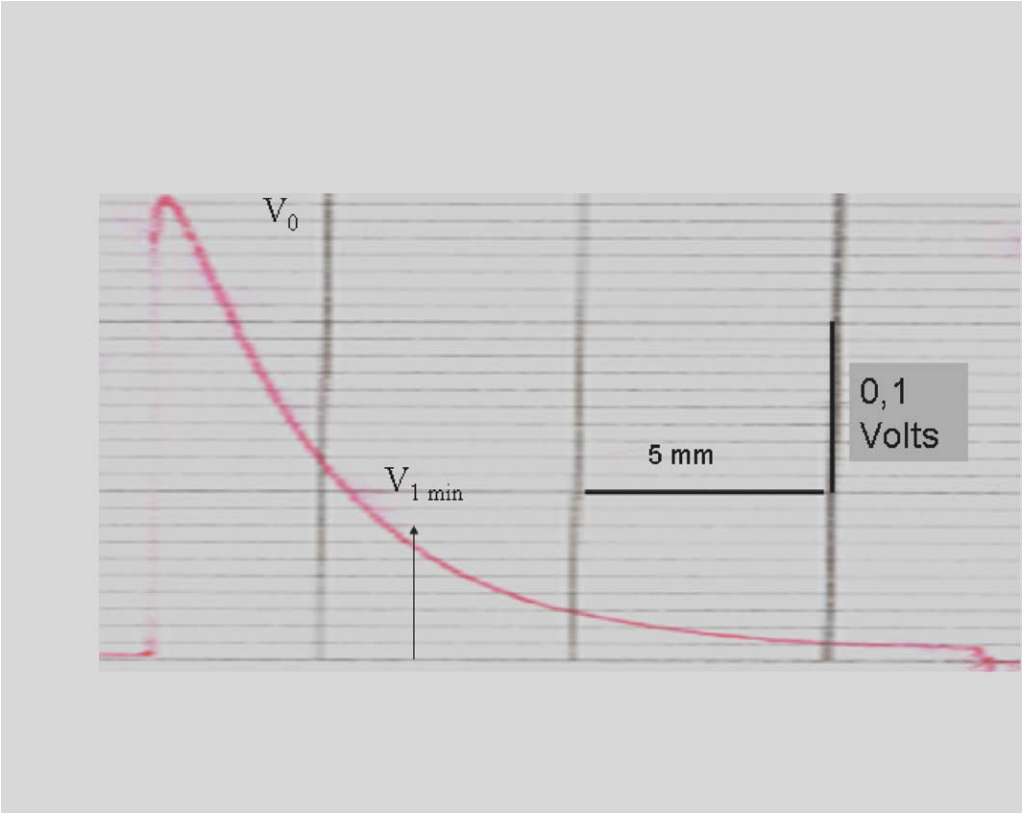

Fig. 1. Example of a graphical recording of the outcoming signal of the luminometer in a control measurement. V_0 (initial luminescence peak) and $V_{1\,min}$ (luminescence after 1 min) are shown. The scale is 5 mm:1 min (x) and 10 mm:0.1 V (y). In this curve we can see an exponential decay in luminescence.

luminescence signals that were used as the base line. V_0 and k were measured and the results were normalised to 100. The standard deviation was 14 for V_0 and normalised to 2. The standard deviation was 0.06 for k.

3.3. ATP luminescence

We measured the luminescence of the luciferine–luciferase reaction with non-irradiated ATP and irradiated ATP at the three ATP concentrations. We found significant differences only at 2×10^{-9} mol/l (Fig. 2).

3.4. Luminescence peak (V_0)

Figs. 3 and 4 show luminescence peaks V_0 normalised to the control value of 100% SEM 11.98. At 2×10^{-9} ATP mol/l concentration, we found significant differences in the luminescence peak V_0 between the control and the irradiated groups at 6 J/cm^2 (138.0 ± 19.42) with the 635 nm laser and at 1 J/cm^2 (130.7 ± 6.26), 4 J/cm^2 (134.6 ± 8.36) and 6 J/cm^2 (153.2 ± 3.47) with the 830 nm laser.

3.5. Reaction rate (k)

We measured the reaction rate constant k at 2×10^{-9} ATP mol/l. The k values normalised to 100% min^{-1} ± 0.96 significantly increased with both wavelengths at 6 J/cm^2 (635 nm 103.5 ± 1.16 and 830 nm 105.79 ± 0.64) and with the 830 nm laser also at 4 J/cm^2 (103.96 ± 1.21) (Fig. 5).

The behaviour of V_0 and k at 2×10^{-10} and 2×10^{-11} mol/l ATP concentration showed no significant differences with the control group.

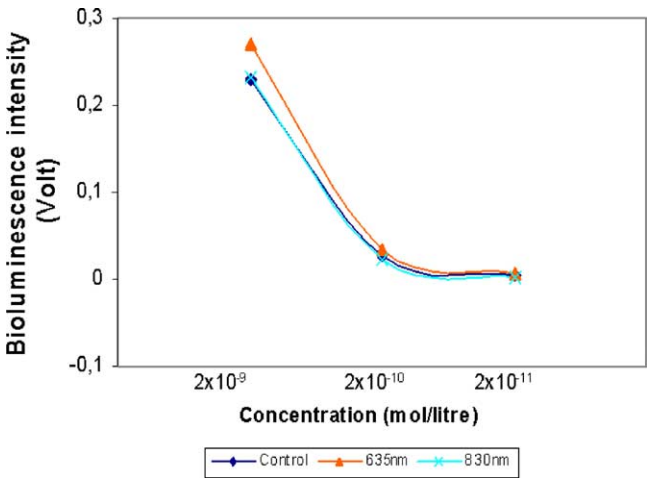

Fig. 2. Luminescence measurement of ATP at 2×10^{-9}, 2×10^{-10} and 2×10^{-11} mol/l in the control groups and with 635 and 830 nm irradiation at 4 J/cm^2. We can see significant changes only at the first ATP concentration. For this reason we used this concentration in all subsequent experiments.

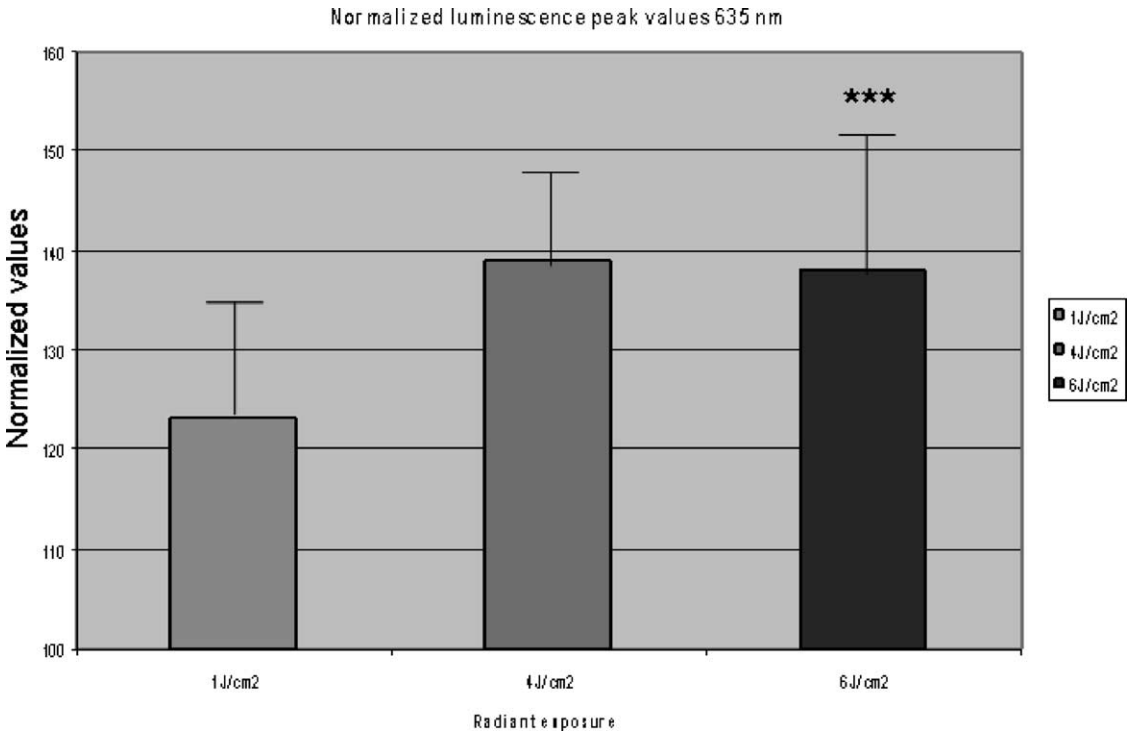

Fig. 3. Normalised luminescence peak V_0 with 635 nm laser irradiation. We achieved statistical significance at 6 J/cm^2 ($^{***}P < 0.0005$). Statistical significance was not reached at the other radiant exposures.

3.6. Area under the luminescence curve

In the control groups, the area under the luminescence curve is normalised at 100% SEM 3.62. With 635 nm, the values for 1, 4 and 6 J/cm^2 were 118.71±11.04, 129.9±7.48 and 113.66 ± 13.68, respectively. With 830 nm, the values were 123.08 ± 5.11, 126.9 ± 7.52 and 142.01 ± 2.8. Fig. 6 shows the normalised values V_0/k. Significant differences were observed at 4 and 6 J/cm^2 with 635 nm and at 1, 4 and 6 J/cm^2 with 830 nm. With 1 J/cm^2 for both wavelengths,

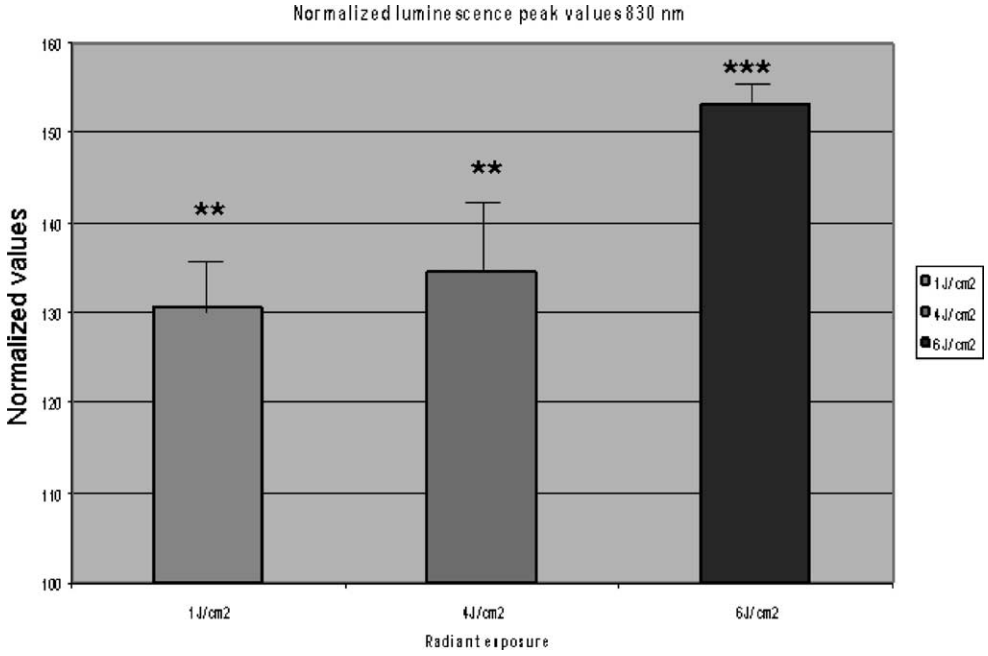

Fig. 4. Normalised luminescence peak V_0 with 830 nm laser irradiation. All irradiated samples were compared with the control. We achieved significance at the <0.001 level with 1 and 4 J/cm^2, and <0.0005 at 6 J/cm^2 ($^{*}P < 0.005$; $^{**}P < 0.001$; $^{***}P < 0.0005$).

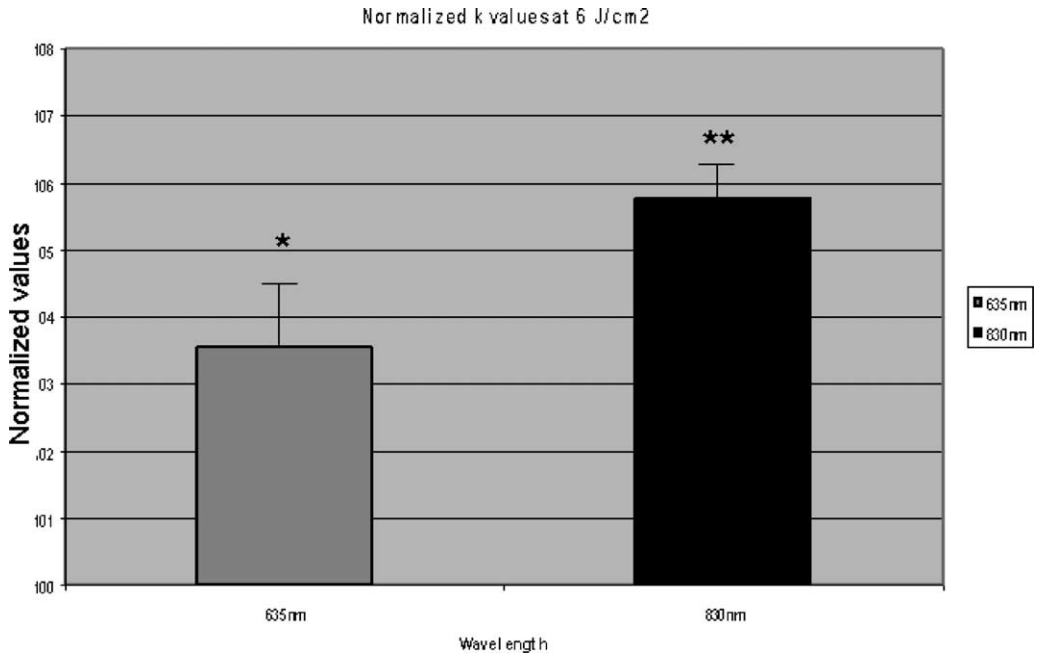

Fig. 5. Normalised k values of the reaction with 635 and 830 nm laser irradiation at 6 J/cm^2. Both lasers at this dose increased the k of this ATP-dependent reaction ($^*P < 0.005$; $^{***}P < 0.0005$).

the difference was 20% greater than with the control. At 4 J/cm^2, the difference was 25%. At 6 J/cm^2, the 635 nm had the same value and the 830 nm increased to 32% (Fig. 6).

3.7. Absorption of ATP

When irradiating ATP with 12 J/cm^2 at 635 nm laser light, we observed a significant increase in absorption compared

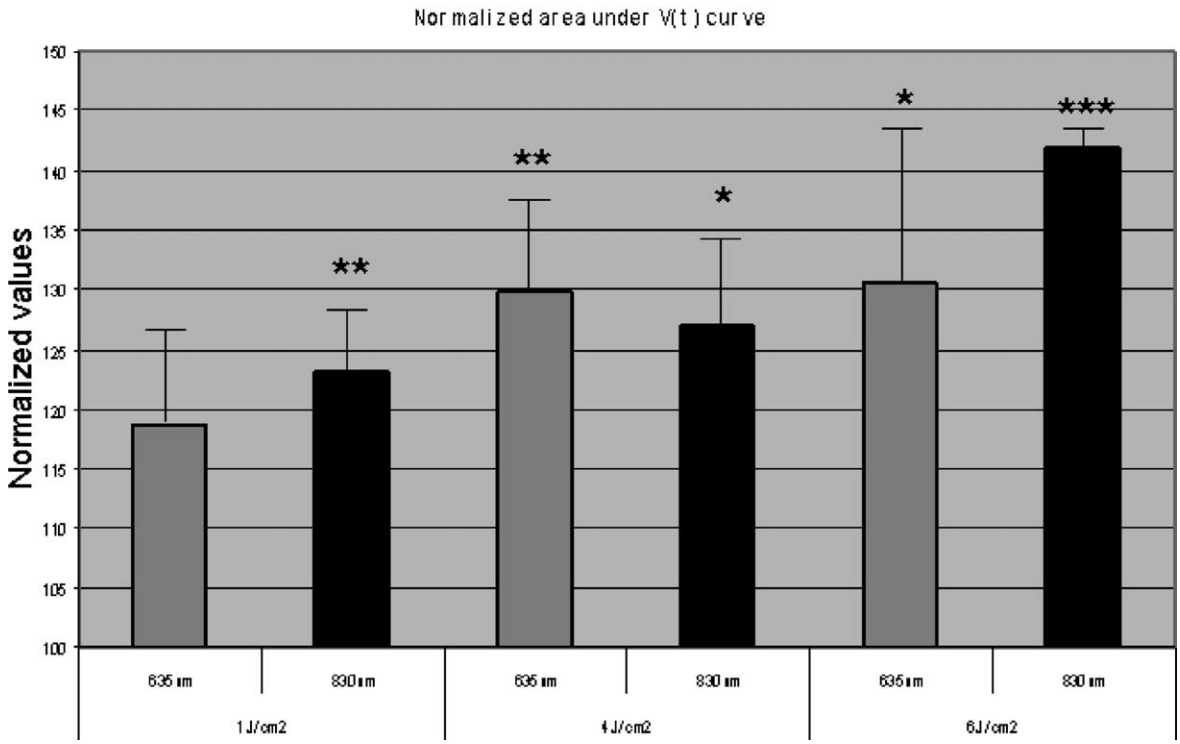

Fig. 6. Normalised areas under the $V(t)$ curve, proportional to the number of ATP molecules that reacted. We achieved statistical significance at 4 and 6 J/cm^2 with 635 nm laser irradiation, and at 1, 4 and 6 J/cm^2 with 830 nm.

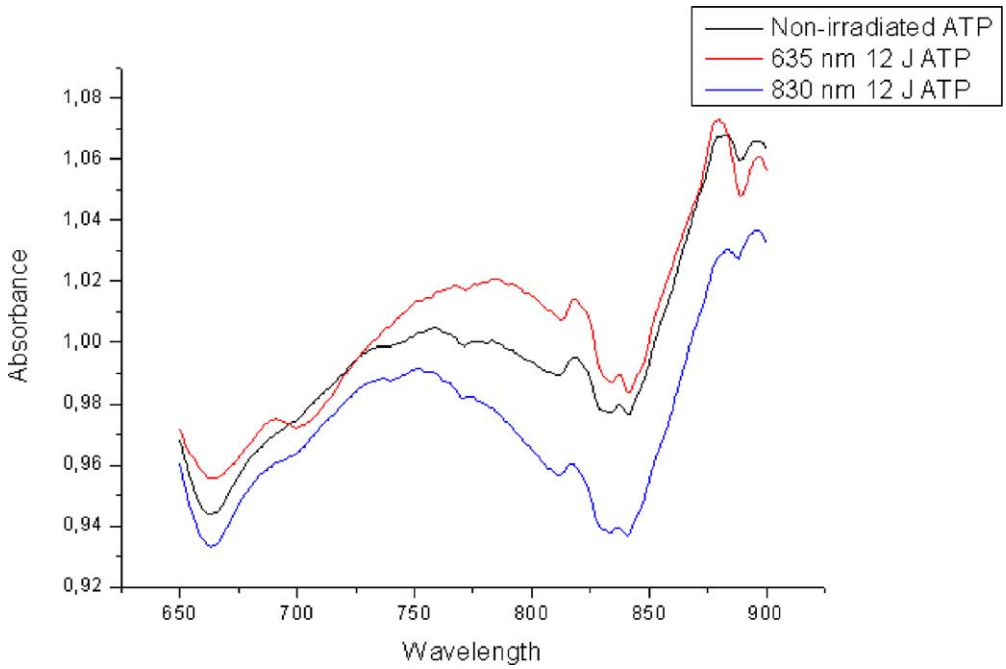

Fig. 7. Irradiated and non-irradiated ATP absorption spectra. In the visible and near-IR wavelengths for ATP irradiation, the absorption spectra changed significantly: 635 nm irradiation changed the band from 790 to 840 nm, and 830 nm irradiation changed the band from 740 to 770 nm.

to non-irradiated ATP from 790 to 840 nm (with P values from 0.004 to 0.048). The near-IR laser at 830 nm changed significantly the 740 to 770 nm band and the irradiated ATP absorbed fewer photons than the control non-irradiated ATP (P between 0.004 and 0.044) (Fig. 7).

4. Discussion

The firefly reaction is a standard model for quantifying ATP concentration based on the reaction:

$$\text{luciferine} + \text{ATP} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{oxiluciferine} + \text{AMP} + \text{PPi} + \text{CO}_2 + \text{luminescence}$$

In our experiments we irradiated ATP at 2×10^{-9} mol/l because we did not obtain a good signal with our equipment at lower concentrations. The initial luminescence peak was proportional to the number of ATP molecules that reacted at the beginning of the process. The larger peak observed after ATP irradiation implies that irradiated ATP has more affinity for the other reagents of the reaction than non-irradiated ATP. The rate constant k measures the rate of decrease in ATP concentration. As k was significantly larger in the irradiated groups, the system reacted faster that normal. We hypothesise that the ATP molecule is electronically excited by the visible light and vibrationally excited by the near-infrared light, and that an excited ATP molecule needs less activation energy to produce its breakdown [2]. The changes found in ATP affinity and in the rate constant must be produced by the total number of molecules that react or by physical and chemical changes in the ATP molecule. We found that more ATP molecules were involved in the reaction after they were irradiated because the area under the luminescence curve was greater. Physical and chemical changes in the molecule are corroborated by the different spectra of irradiated and non-irradiated ATP from 740 to 840 nm. Another interesting observation is that the increase in V_0 and k was not proportional to the radiant exposure. We hypothesise that some form of saturation of ATP molecules may have occurred.

These results can provide new information about primary mechanisms in photobiology and photomedicine, such as the effects observed in low level laser therapy (LLLT).

5. Conclusions

Our results show there were significant differences between the non-irradiated ATP controls and the irradiated ATP groups. The initial luminescence peaks and the k-values were greater in the irradiated groups. Also, the area under the luminescence curve and the absorption spectra confirm a physical and chemical change in irradiated ATP molecules.

Acknowledgements

The authors would like to thank SOR International SA for supplying the GaAlAs laser used in this study, and

Dr. J. Massons (Física Aplicada, Facultat de Quimica, Universitat Rovira i Virgili) for his help in laser power measurement.

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