Original Research

Evaluation of the photobiomodulation in L929 cell culture

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Abstract

Low-level laser therapy has become an important tool for bio-modulation process. It can induce stimulatory or inhibitory effects according to cell behavior at specific irradiation. Our objective was to determine L929 cell line response to irradiation at λ 685 and 830 nm, concentrations of 5 and 10% fetal bovine serum and different energy densities of 0.1, 0.5, 1, 2, 3, 5, 7, 10, 20, and 30 J/cm^2. Thus, cells were plated at 1×10^5 cells/mL and irradiated with semiconductor laser As-Ga-Al. Twenty-four hours after irradiation, cells were subjected to MTT, neutral red, crystal violet tests, and cell staining was performed using the kit Alexa Fluor 488 Annexin V-FITC and propidium iodide. Our results showed that low-level laser therapy stimulates effect when the energy density is 5 to 3030 J/cm^2 and inhibits effects on energy density 0.1 to 3 J/cm^2. This inhibitory effect was evidenced by the absence of dead cells labeled, decreased cell density, and by the absorption of neutral red in intact cells. The study also demonstrated that fetal bovine serum, at different concentrations, did not affect response of the cells after irradiation.

Keywords: Bio-modulation, low-power laser, proliferation, inhibition

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Introduction

After the discovery that the biological systems are sensitive to light, great interest has been aroused in the scientific community, generating research and technological development in different directions, including basic and applied science. A strong boost to this idea was given by the introduction of laser as a light source that offers many benefits,[1,2] thus increasing the demand for a better understanding of its cellular and molecular effects.

Low-level laser therapy (LLLT) was developed between 1960 and1970[3] and consists of a type of photo-modulation whose effects are biochemical and not thermal and, therefore cannot harm living tissues at the cellular level.[4]

Primary responses to radiation are not fully understood, but it is believed that the key event is the excitation of the components of the mitochondrial respiratory chain. Subsequently, numerous signaling cascades in the cytoplasm, membranes and nuclei are active, characterizing the secondary responses.[5–7] This chain of cellular signaling results in proliferative effects,[8,9] inhibitory effects,[4,10] increased cell viability,[11] morphology alteration, and cellular organelles,[12,13] activation of proteins[14,15] among others.

The aim of this study was to evaluate the effect of LLLT (wavelength λ 685 and 830 nm, semiconductor laser As-Ga-Al) in L929 fibroblast cultured with different concentrations of fetal bovine serum (FBS). Our results showed that under the experimental conditions used, LLLT can exert not only proliferative effects but also inhibitory effects.

Materials and methods

Cell culture

L929 cells, derived from mouse conjunctive tissue (ATCC CCL-1 NCTC), were obtained from the Cell Culture Laboratory of the Instituto Adolfo Lutz (São Paulo, SP, Brazil) and were cultured in minimal essential medium (MEM – Gibco® – Life Technologies) supplemented with 10% FBS (Gibco® – Life Technologies) and 1% of antibiotics (antibiotic selection – Gibco® – Life Technologies). Cells were maintained at 37°C and 5% CO_2.

Irradiation with low-power laser

Before the irradiation, medium was removed and Hank's balanced salt solution (Sigma-Aldrich®) was added. Cells were irradiated with a portable clinical device with semiconductor laser with an active medium, indium phosphide-gallium-aluminum (InGaAlP – Thera Lase – DMC, São Carlos, Brazil) operating in a continuous mode (continuous wave – CW), a power output of 30 mW, spot size of 0.5 cm^2 and an output density of 60 mW/cm^2 for both wavelengths. The control group (5% FBS) and control (10% FBS) were free

of any treatment. The parameters used for the irradiation are shown in Table 1.

After irradiation, cells were incubated with 5 or 10% FBS in culture conditions. All analyses were performed 24 h after irradiation.

Cell viability assay – MTT

The mitochondrial activity was assessed by the MTT cytotoxicity assay (3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide). Twenty-four hours after the irradiation, cells were incubated with 5 mg/mL MTT (Sigma-Aldrich®, Steinheim, Germany) for 2 h at 37°C. Subsequently, the solution was removed and dimethylsulfoxide (DMSO – Sigma-Aldrich®, Steinheim, Germany) was added for 20 min under stirring. After this period, plates were read with a 570-nm filter in an ELISA Spectracount Reader (Packard Instrument Company Inc., Meriden, CT, USA).

Neutral red assay

Lysosomal activity was assessed by neutral red assay (NR). After the laser therapy, cells were incubated for 2 h with the dye NR (Sigma-Aldrich®, Steinheim, Germany) at a concentration of 100 mg/mL. Then the solution was removed and ethyl alcohol/acetic acid/distilled water was added at a concentration of 50:1:49%. The plate was subjected to stirring for 20 min and subsequently was read with a 570-nm filter in an ELISA Spectracount Reader (Packard Instrument Company Inc., Meriden, CT, USA).

Crystal violet assay

In order to evaluate cell density after irradiation, cells were subjected to crystal violet (CV) test. Treated cells were incubated with staining solution (5% VC, 1.7% NaCl, 3.3% paraformaldehyde, 33.3% ethanol, and 56.7% water) for 3 min at room temperature. Cells were then washed and subsequently 1% Sodium Dodecyl Sulfate solution (SDS – Sigma-Aldrich®, Steinheim, Germany) was added. Cells were kept for 1 h at room temperature and the plates were read with a 570-nm filter in an ELISA Spectracount Reader (Packard Instrument Company Inc., Meriden, CT, USA).

Table 1 Parameters used in LLLT AsGaAl (see details in Materials and methods section)

Energy density, fluence (J/cm^2)	Irradiation duration (s)	Wavelengt (nm)	Concentration of FBS (%)
0.1	2	685 and 830	5 and 10
0.5	9	685 and 830	5 and 10
1	17	685 and 830	5 and 10
2	34	685 and 830	5 and 10
3	51	685 and 830	5 and 10
5	84	685 and 830	5 and 10
7	117	685 and 830	5 and 10
10	167	685 and 830	5 and 10
20	334	685 and 830	5 and 10
30	501	685 and 830	5 and 10

Fluorescence microscopy – apoptosis and/or necrosis

In order to verify the presence or absence of cell death 24 h after irradiation, cells were subjected to the test using Annexin V/Alexa Fluor® 488 and propidium iodide (PI) (Invitrogen™ kit – Sigma-Aldrich®, Steinheim, Germany). Twenty-four hours after irradiation, cells were stained with 50 μL of Annexin V/Alexa Fluor® 488 solution and PI (100 mg/mL) for 15 min at room temperature. The solution was removed and cells were washed with 1× binding buffer. After labeling, cells were fixed with paraformaldehyde (Sigma-Aldrich®, Steinheim, Germany) 4% for 10 min and washed with phosphate buffered saline. Observations were conducted in DMLB Leica fluorescence microscope and images captured via digital video camera Leica DFC 300 FX.

Statistical analysis

Statistical calculations were done using GraphPad Prism® statistical data analysis software version 5.01 – 1992–2007. Data were compared by analysis of variance followed by Tukey test, setting the significance level of 5% ($p \leq 0.05$). Experimental data, mean, and standard deviation are indicated. All experiments were performed in quadruplicate with two independent experiments.

Results

Cell viability by MTT assay

The viability of the treated cells had a small significative reduction ($p \leq 0.05$) when irradiated at λ 685 nm, 5% FBS, and energy densities (ED) 0.1 J/cm^2 (Figure 1(a)).

When irradiated at higher ED (5–30 J/cm^2), cellular viability had a significative increase ($p \leq 0.05$) with 5% FBS at λ 685 nm ED 20 J/cm^2 (Figure 1(a)) and λ 830 nm ED 10 J/cm^2 (Figure 1(c)).

When irradiated with the concentration of 10% FBS, there were no changes in mitochondrial activity at λ 685 nm (Figure 1(b)) and λ 830 nm (Figure 1(d)).

Cell viability by NR assay

The NR assay aims to check the survival and cell viability based on the ability of viable cells to incorporate the NR dye by lysosomes, which accumulates in its entirety membrane.[16]

Figure 2(a) (λ 685 nm and 5% FBS), 2(b) (λ 685 nm and 10% FBS) and 2(c) (λ 830 nm and 5% FBS) shows that the NR concentration was not increased in irradiated groups compared to control. However, a significant increase ($p \leq 0.05$) in the uptake of ED was observed at 0.5, 1, 2, and 3 J/cm^2 and a decrease when ED was 10, 20, and 30 J/cm^2 at λ 830 nm and 10% FBS (Figure 2(d)).

Cell density by CV assay

Cell density was significantly reduced ($p \leq 0.05$) when irradiated at λ 685 nm, 5% FBS, and ED 0.1 to 2 J/cm^2

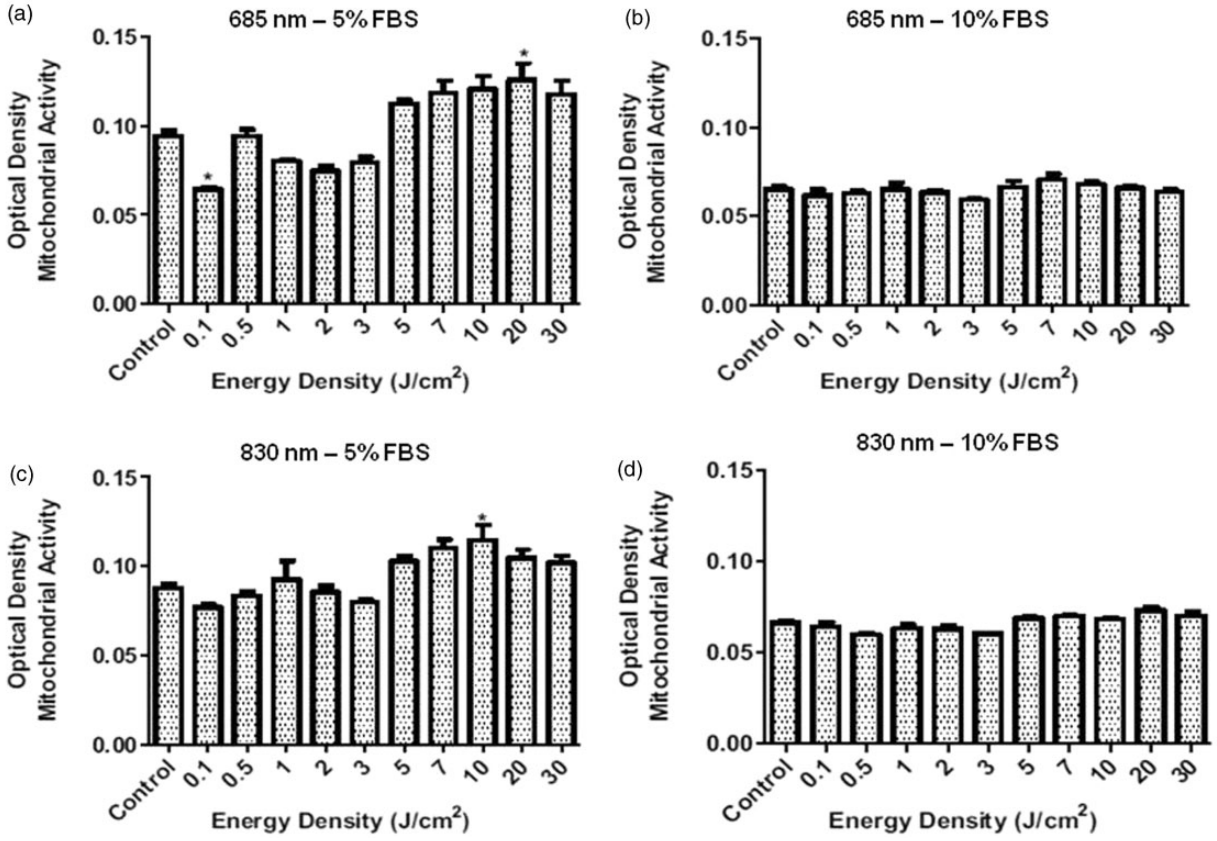

Figure 1 Data representing the response of L929 line after irradiation with LLP and MTT assay. (a) λ 685 nm and a concentration of 5% FBS; (b) λ 685 nm and concentration of 10% FBS; (c) λ 830 nm and a concentration of 5% FCS; (d) λ 830 nm and concentration of 10% FBS. $^{*}p \leq 0.05$

(Figure 3(a)). At λ 685 nm and 10% FBS, there was a significant reduction ($p \leq 0.05$) in cell density in ED 0.1 to 3 J/cm^2 compared to control group (Figure 3(b)).

The same behavior was observed at λ 830 nm and 5% FBS. The ED of 0.1 to 0.5 J/cm^2 indicated a significative decrease ($p \leq 0.05$) in cell density. In ED from 7 to 30 J/cm^2, a significant increase ($p \leq 0.05$) in cell density was observed compared to the control group (Figure 3(c)). At λ 830 nm and 10% FBS, a significative reduction ($p \leq 0.05$) in cell density was observed for ED of 1 and 3 J/cm^2 and a significant increase ($p \leq 0.05$) of 5 to 30 J/cm^2.

Cell death detection

In order to study whether the treatments were able to activate cell death mechanisms, cell staining was performed with PI and Annexin V in irradiated cultures compared with control group. For both groups, control and irradiated, the label obtained was similar, even for the different experiments used. According to our data, neither different concentrations of FBS, ED nor wavelength were able to increase apoptosis or necrosis.

Discussion

Stimulation of proliferation

Over the years, many studies have demonstrated the effectiveness of LLLT as a tool to stimulate proliferation. This effect has been described in osteoblasts[17] and human gingival fibroblasts.[18] In this study, we demonstrated the stimulation of proliferation when λ 830 nm at both concentrations of FBS (5 and 10%), and ED 5 to 30 J/cm^2 (Figure 3(c) and (d)).

Inhibition of proliferation

In addition to proliferative effects, studies have also demonstrated that LLLT is capable of promoting the inhibition of proliferation in lymphocytes,[19] hematopoietic cell lineages,[20] breast cancer,[21] mesenchymal cells.[10]

Our results showed that, for both wavelengths (λ 685 and 830 nm) and both concentrations of FBS (5 and 10%), cells reduced their density ED from 0.1 to 3 J/cm^2 (Figure 3(a) to (d)). It is suggested that this reduction has occurred due to the inhibition of proliferation induced by irradiation, since necrosis and apoptosis markers were not labeled. Furthermore, the fact that cells incorporate the NR after irradiation in both wavelengths (λ 685 and 830 nm) and both concentrations of FBS (5 and 10%) suggests absence of cell death and lysosomal membrane integrity indicating the proper functioning of the protons pump in the irradiated cells,[22] as shown in Figure 2(a) to (d).

Although it seems controversial, the decreased lysosomal activity in ED 10, 20, and 30 J/cm^2 (Figure 2(d)) compared to the increased cell density with the same parameters, it has to be considered that LLLT acts

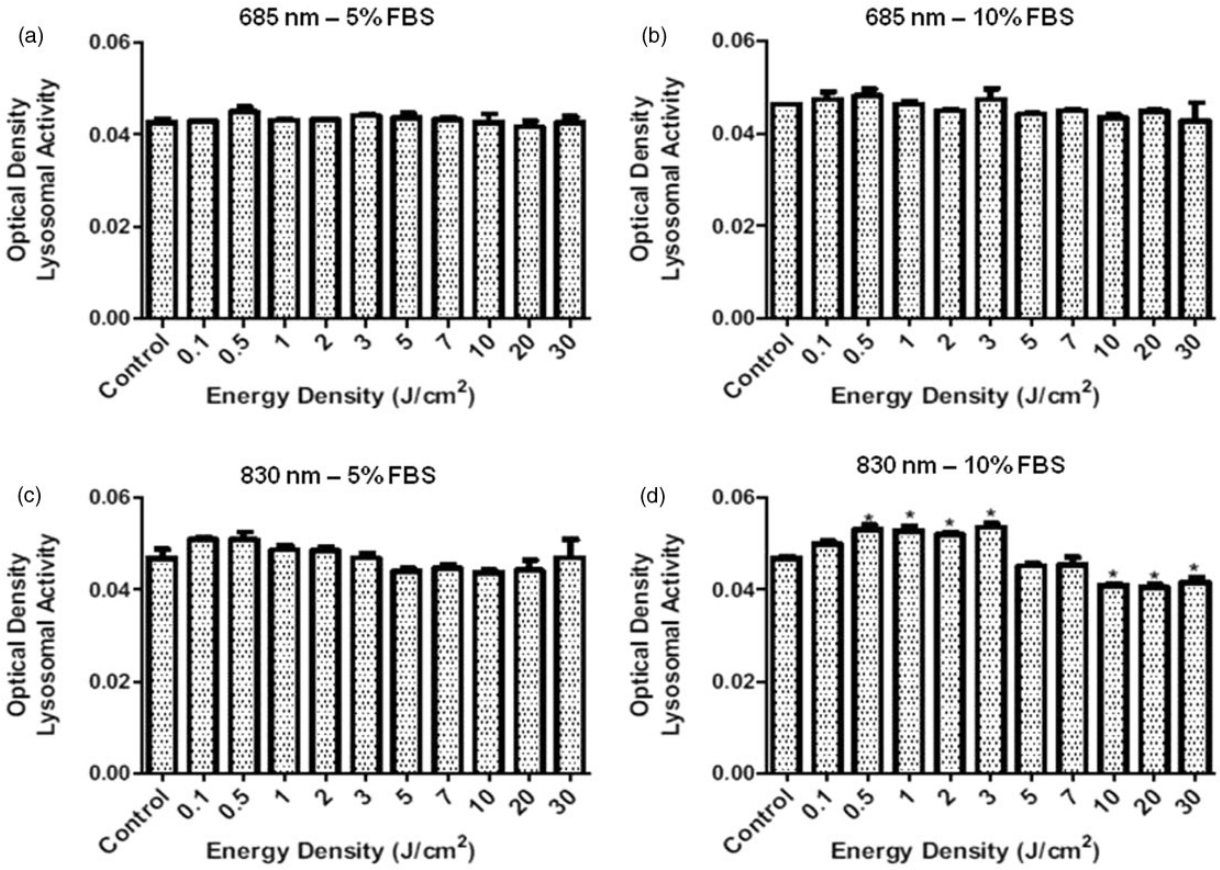

Figure 2 Data representing the response of L929 line after irradiation with LLP and the NR test. (a) λ 685 nm and a concentration of 5% FBS; (b) λ 685 nm and concentration of 10% FBS; (c) λ 830 nm and a concentration of 5% FCS; (d) λ 830 nm and concentration of 10% FBS. $^{*}p \leq 0.05$

differently in cell organelles and cell responses differently at the irradiation moment, dictating the response after therapy.[3]

LLLT, FBS concentration, and wavelength

It has been reported that irradiation exerts effect on stressed cells or on cells with altered metabolism. The irradiation function would be to normalize cell changes, returning to its ideal condition.[23] Studies have shown that one of the ways to stress cells *in vitro* is by reducing FBS concentration in cell culture medium, because the growth rate of cells under these conditions is lower compared with the growth in ideal conditions.[9]

Eduardo *et al*.[24] demonstrated that LLLT is capable of inducing cells proliferation even under serum deficit, although best results were obtained under normal culture conditions.

Our results demonstrated greater stimulation of cell growth at a concentration of 5% FBS in both wavelengths (Figures 1(a), 1(c), and 3(c)). However, this difference was not significant compared to the treated groups at the concentration of 10%. We suggest that the lower stimulation of cell is due to the small nutritional deficit induced in this study, causing it to respond similar to irradiation at a concentration of 10% FBS. However, further studies should be conducted in larger conditions of nutritional deficit and using the same parameters proposed in this paper in order to verify if LLLT is able to normalize cellular metabolism or if the irradiation is more efficient under normal growing conditions.

In relation to the wavelength, it was observed that the λ 830 nm was more efficient for cell proliferation since an increase of 33% was indicated for groups 5% FBS and 22% for both groups with 10% FBS compared with the λ 685 nm (16% for both FBS concentrations).

Karu[23] describes that the short-term effect of LLLT is due to the mitochondrial stimulation, whereas the long-term effects are stimulant or inhibitory for DNA transcription and replication. These responses may be a result of the immediate effects that occur during or after irradiation, or long-term effects, which can occur hours or days later.[8,25] Thus, further studies are necessary in order to evaluate the behavior of L929 strain under the parameters proposed in this work and at different periods of irradiation.

Conclusion

The present study demonstrated that LLLT has proliferative and inhibitory effects on L929 cells, depending on the

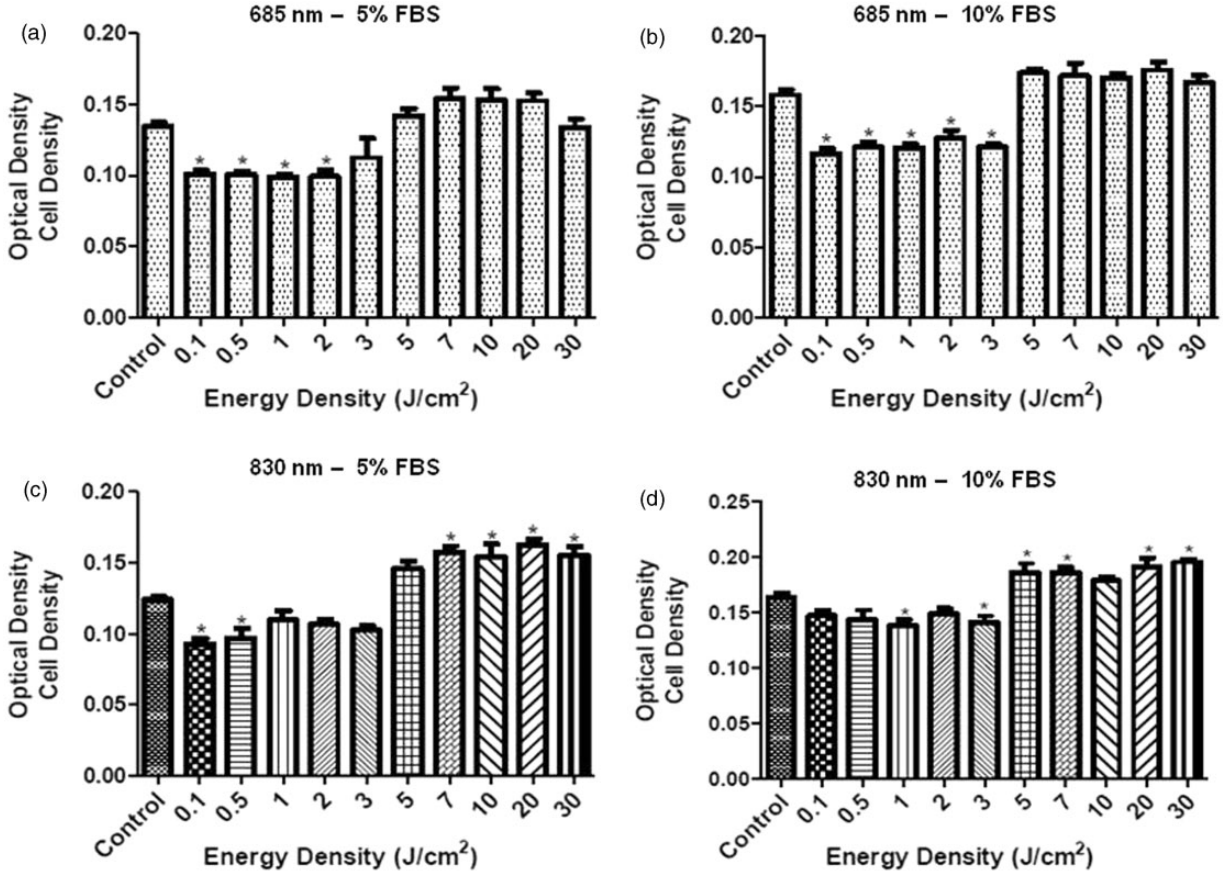

Figure 3 Data representing the response of L929 line after irradiation with LLP and CV testing. (a) λ 685 nm and a concentration of 5% FBS; (b) λ 685 nm and concentration of 10% FBS; (c) λ 830 nm and a concentration of 5% FCS; (d) λ 830 nm and concentration of 10% FBS. *$p \leq 0.05$

parameters chosen for irradiation, which influence the results. It was also demonstrated that the metabolic condition of the cells dictates the intensity response after irradiation.

AUTHORS CONTRIBUTION

JMM – lead author; this manuscript is part of her dissertation (Masters degree). CPS – manuscript writing and review; assistance with cell line maintenance. NSDS – manuscript writing and review; assistance in developing experimental groups.

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