Genotoxic effect of photodynamic therapy mediated by curcumin on *Candida albicans*

Runing Title: Curcumin-mediated PDT-induced DNA damage on *Candida albicans*

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ABSTRACT

Photodynamic therapy (PDT) is a promising method for localized and specific inactivation of fungi and bacteria. A nontoxic light-sensitive compound is taken up by cells, which are then exposed selectively to light, which activates toxicity of the compound. We investigated the potential of sub lethal photodynamic therapy (PDT) using light sensitive curcumin (CUR) in combination with blue (455 nm) light to promote reactive oxygen species (ROS) formation in the form of singlet oxygen and DNA damage of *Candida albicans*. Surprisingly, CUR-mediated PDT but also light alone caused significantly longer comet tails, an indication of DNA damage of *C. albicans* when compared with the negative control. The intracellular ROS production was also significantly higher for the group treated only with light. However, PDT compared to blue light alone significantly slowed DNA repair. Comet tails decreased during 30 min visualized as a 90% reduction in length in the absence of light for cells treated with light alone, while comet tails of cells treated with PDT only diminished in size about 45%. These results indicate that complex mechanisms may result in PDT in a way that should be considered when choosing the photo-sensitive compound and other aspects of the treatment design.

Keywords: Photoinactivation, fungi, photosensitizer agent, genotoxicity.

INTRODUCTION

The species of *Candida* genus are considered as commensal microorganisms that are part of the oral microflora of healthy individuals. Under certain circumstances, these microorganisms can act as opportunistic pathogens, being responsible for the development of infections. Oropharyngeal candidiasis (OPC) has been reported as the most common opportunistic infection caused by an accentuated growth and penetration of fungal species in the oral tissues (Canon *et al*., 1995; Pfaller & Diekema, 2007). Some predisposing factors such as poorly fit dentures, AIDS, and broad-spectrum antibiotic use over extended periods of time can enhance host susceptibility to develop this infection (Samaranayake, 1990; Scully *et al*., 1994; Shapiro *et al*., 2011). Disseminated candidiasis in immunocompromised patients can cause high mortality rates (Kromery & Barnes 2002; Pfaller & Diekema, 2007). Treatments used against infections caused by *Candida* spp. are routinely based on the use of

medications that may be topical or systemic (Samaranayake, 1990; Pappas *et al*., 2009). However, the use of standard antifungal therapy may be limited due to its toxicity, low efficacy, or microorganism resistance after medication exposure over extended periods of time (Perezous *et al*., 2005). Thus, studies have been conducted in an endeavor to find alternative strategies to inactivate *Candida* species. Within this context, photodynamic therapy (PDT) has been suggested for inactivation of some viruses (Wainwright, 2004), bacteria (Gois *et al*., 2010) and fungi (Dovigo *et al*., 2011a, c).

The use of PDT therapy for treating non-oncological diseases, including microbial inactivation and infection treatments, is still a recent field of scientific investigations (Konopka & Golinski, 2007; Donnelly *et al*., 2008). The photodynamic process requires the use of a photosensitizing agent (PS), light application that corresponds to the absorbance spectrum of the PS and the presence of oxygen (Wainwright, 2004; Jori *et al*., 2006). Photosensitization results in reactive species capable of inducing cell inactivation (Bonnett & Martínez, 2001). It has been suggested that the mechanism involves absorption of photons by the PS, which lead electrons to an excited state. In the presence of oxygen, light-excited PS can react with neighboring molecules through the transfer of electrons or hydrogen (type I reaction) or by energy transfer to oxygen (type II reaction), culminating to the production of reactive oxygen species (ROS) (Bonnett & Martínez, 2001). These ROS have non-specific reactivity with organic molecules. This means that any cellular macromolecule may be a potential target for PDT (Henderson & Dougherty. 1992; Bonnett & Martínez, 2001). Therefore, PDT seems to have a significant advantage over conventional antifungal treatments, since the plurality of targets decreases the likelihood of development of resistance of the exposed microorganisms in this procedure.

The inactivation of *Candida* spp. has been discussed in several studies (Sonis *et al*., 2000; Lambrechts *et al*., 2005; Konopka & Golinski, 2007; Junqueira *et al*., 2009), however it has been reported that it is more difficult to eliminate this type of fungus than gram-positive bacteria (Lambrechts *et al*., 2005). According to Demidova & Hamblin (Demidova & Hamblin, 2005), the presence of the nuclear membrane, the greater fungal cell size, and the reduced number of targets for singlet oxygen per unit volume of cell require a higher concentration of photosensitive drug and higher light doses to inactivate the fungus. Furthermore, the similarity of the fungal cell with mammalian cells complicates the selective accumulation of the photosensitive drug in the fungus (Donnelly *et al*., 2008).

Different types of PSs have been proposed in *in vitro* studies, including porphyrins (Bliss *et al*., 2004; Dovigo *et al*., 2011b), phenothiazines (Paardekopper *et al*., 1992; Zeina *et*

al., 2001), chlorins (Strackhovskaya *et al*., 2002; Copley *et al*., 2008) and phthalocyanines (Mantareva *et al*., 2011). Additionally, another PS that has been studied in anti-cancer therapy is curcumin (CUR), a yellow-colored compound extracted from the rhizome of *Curcuma longa L* (saffron) (Sharma *et al*., 2005). Several studies suggest that CUR has anti-inflammatory, antioxidant, and immunomodulatory effects, which could be exacerbated by light at a suitable wavelength (Bruzell *et al*., 2005). Recently, PDT mediated by CUR associated with light emitting diode (LED) was effective in inactivating planktonic suspensions and biofilms of *C. albicans*, *C. glabrata*, and *C. dubliniensis* (Andrade *et al*., 2013). CUR has also shown an antifungal effect on the inactivation of biofilms and cell suspension cultures of clinical isolates of *C. albicans*, *C. glabrata* and *C. tropicalis*, promoting a reduction in cellular metabolism by 85, 85, and 73%, respectively (Dovigo *et al*., 2011a). The compound was effective for the inactivation of *C. albicans* present in the tongue of mice with induced oral candidiasis, promoting an approximate 5-$\log_{10}$ reduction in cell viability, without causing any damage to the tongue tissues of the animal (Dovigo *et al*., 2013).

It is important to point out that there are no studies in the literature reporting that PDT, PS or light alone can be genotoxic to fungal cells. Damage can be the result of various processes and agents of endogenous or exogenous origin that may induce mutagenic processes (Henle *et al*., 1996). Examples of damage are double-strand breaks or oxidized bases that can lead to single strand breaks with subsequent DNA relaxation, increasing its electrophoretic mobility. The extent of DNA damage can be assessed by single-cell gel electrophoresis, also known as comet assay, where the chromosomal DNA migration distance correlates with the extent of damage of the DNA in low melting agarose-embedded cells (Azevedo *et al*., 2011). The comet assay has been used in a number of different applications, such as testing for genotoxicity, ecological monitoring, and human biomonitoring (Collins, 2004). To our knowledge among the studies that evaluated this type of test, only one was performed in yeasts of the genus *Candida* (Farrell *et al*., 2011). The explanation for this fact is that the amount of DNA per cell in these microorganisms is considerably lower than in other eukaryotic cells, which could make the visualization of the comet difficult (Rank *et al*., 2009).

According to the above-mentioned aspects, the mechanism of action of PDT is based on the production of ROS, which are responsible for causing cell damage through oxidative stress. ROS production, such as superoxide radical ($^{\cdot}O_2^-$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen constitute a significant threat to cellular integrity (Henle

et al., 1996). It has been suggested that the presence of redox-active metal ions such as Fe^{2+}, oxygen, and H_2O_2 can lead to Fenton chemical reaction, culminating in the production of highly reactive radicals to the cellular components. Oxidative stress can cause oxidative damage to macromolecules such as the DNA (Boiteux & Guillet, 2004), which contributes to genetic instability and possible mutations (Henle *et al*., 1996). Thus, determining DNA damage by the comet assay would provide information on the genotoxic potential of PDT and/or of its components (PS and light) due to oxidative stress caused on cells. Currently, CUR has shown promising results in the inactivation of *Candida* spp. Thus, the aim of this *in vitro* study is to assess the potential of PDT mediated by CUR in causing DNA damage on *C. albicans* cells.

MATERIAL AND METHOD

Analysis of DNA damage of *C. albicans* cells

Photosensitizing agent and light source

For the experiments we used natural CUR (Sigma-Aldrich, St. Louis, MO). A stock CUR solution (600 mM) was prepared in 10% dimethyl sulfoxide and then diluted in saline to obtain the concentrations to be tested. The blue LED device was developed by the Institute of Physics of São Carlos (University of São Paulo, São Carlos, SP, Brazil) and consisted of a handpiece (LXHL-PR09, Luxeon1 III emitter, Lumileds Lighting, San Jose, CA) that provided uniform emission of 440-460 nm with light energy equivalent to 89.2 mW/cm^2.

Microorganisms and growth conditions

All assays were performed on the type strain *C. albicans* ATCC 18804, a clinical isolate in Uruguay purchased from ATCC (American Type Culture Collection; www.atcc.org/). Fungal cells used in the study were cultured in YPD medium (1% w/v yeast extract, 2% w/v peptone and, 2% w/v dextrose) at 37ºC, 200 rpm and frozen at -70°C. Prior to the experiments, cultures of yeast were grown aerobically at 37°C for 48 h on solid YPDA medium (YPD with 2% w/v agar).

Planktonic cultures of *Candida albicans* and PDT

To conduct the study, a pre-culture of *C. albicans* was prepared in a tube containing 5 ml of 0.67% (w/v) YNB medium (Yeast Nitrogen Base; Difco) supplemented with 2% (w/v) glucose, which was incubated in an orbital shaker at 37°C at 200 rpm for 16 h. After incubation, standardized suspensions of *C. albicans* were obtained by diluting the culture and further incubation for two generations time until mid-exponential growth phase (0.4 optical density at 600 nm, which corresponds to 10^6 cells/ml). The cells were then centrifuged at 18,000 x*g*, washed twice for 2 min and suspended in lyticase buffer (1.0 M sorbitol, 25 mM KH_2PO_4, 50 mM β-mercaptoethanol, and 500 U/ml lyticase (Sigma-Aldrich, Ireland, pH 6.5). The suspension was incubated at 30°C for 30 min. After this incubation, the cells were centrifuged (18,000 x*g*) and washed twice with S buffer (1.0 M sorbitol and 25 mM KH_2PO_4, pH 6.5).

To perform PDT, 100 µL aliquots of the suspension were placed in 96-well microplates and the experimental groups were determined. The samples of the group treated with PDT were exposed to 2.5 µM CUR for 20 min in the dark (pre-irradiation time) followed by illumination for 7 min, giving a total light irradiance of 37.5 J/cm^2 (group P+L+). The effect of the application of isolated PSs (group P+L-) was performed by photosensitization of the samples in 2.5 µM CUR, which remained at rest in the dark for 20 min and then left on the laboratory bench for 7 min (time equivalent to the light dose applied to samples P+L+). To assess the effect of light alone (P-L+), the microplate was left at rest in the dark for 20 min and subsequently illuminated by the device at the light dose proposed (37.5 J/cm^2). Additionally, we assessed the negative control group (P-L-) that was composed of cells that received no treatment. The H_2O_2 group consisted of samples treated with 10 mM H_2O_2, which was considered as positive control since this substance promotes the formation of comet tails in *C. albicans* cells. Thus, five experimental conditions were performed on three different occasions.

Comet assay

After the established treatments, each sample was removed from the microplate wells and placed in microtubes, which were centrifuged again at 18,000 x*g* for 2 min. Then, the supernatants were discarded and the pellets were resuspended in 100 µL of 1.5% (w/v in S buffer) low-melting agarose (LMA) at 30ºC, placed on glass slides, covered with coverslips and incubated in at 4°C for 10 min for gelation of agarose. After this period, the coverslipswere removed and the slides were first immersed in lysis buffer (30 mM NaOH, 1

M NaCl, 50 mM ethylenediamine tetraacetic acid (EDTA), 10 mM tris-HCl, 0.05% (w/v) lauroylsarcosine, pH 10) for 20 min, followed by electrophoresis buffer (30 mM NaOH, 10 mM EDTA, 10 mM Tris-HCl, pH 10) for 20 min. Electrophoresis was performed at 0.7 V/cm for 10 min, the slides were submerged in neutralization buffer (10 mM Tris-HCL, pH 7.4) followed by fixation in 96% methanol for 10 min at room temperature and then they were air-dried. The samples were stained with GelRed™ (Biotium Uniscience) and then analyzed by fluorescence microscopy at 400X magnification, equipped with excitation filter of 475-490 nm. At least 50 representative images of each of the glass slide were analyzed with the help of the free edition of the CometScore™ software version 1.5 (free access at http://autocomet.com/products_cometscore.php). The analytical parameter tail length (in µm) was chosen as the unit of DNA damage, corresponding to the head diameter of the comet subtracted from the total length. Thus, the occurrence of DNA damage was identified by the formation of stained comets, unlike intact DNA that presented circular morphology (Azevedo *et al*., 2011).

Analysis of intracellular ROS production

The test was conducted to assess the amount of ROS produced by the groups tested using the fluorescent probe 2',7'-dichlorofluorescein diacetate (H_2DCF-DA; Life Technologies, San Francisco, CA, USA). For this purpose, a 5 mL cell suspension of *C. albicans* was centrifuged once at 18,000 x*g* for 5 min and resuspended in ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). Then, 500 µL of untreated cells were removed for evaluation of autofluorescence and kept in the dark at 4ºC (AF group). H_2DCF-DA (50 µM) was added to the remaining cell suspension and incubated at 37°C for 1 h in the dark. The cells were washed twice with the same amount of PBS and 500 µL aliquots were distributed to the different experimental conditions treatment with PDT (P+L+), only light (P-L+), only CUR (P+L-) or 10 mM H_2O_2. The control consisted of H_2DCF-DA-loaded cells without any treatment (P-L-). After treatments, the cells were washed twice with PBS and quantification of ROS was analyzed by fluorimetric test using Fluoroskan (Thermo Scientific Fluoroskan Ascent- Thermo®) at 495-527 nm. Immediately after the reading, *C. albicans* cells were placed on round glass slides and fixed in 4% paraformaldehyde for 30 min at 37°C, and then analyzed by fluorescence microscopy.

Analysis of DNA repair in *C. albicans* cells

To assess the ability of DNA damage repair after the treatments proposed, analysis of DNA damage was performed using the comet assay, as described above. For this, *C. albicans* cells were centrifuged at 18,000 x*g* for 2 min and resuspended in 100 µL S buffer. The samples were incubated at 37°C for different time intervals of up to 30 min to allow DNA repair. Then they were centrifuged at 18,000 x*g* at 4°C for 2 min and embedded in LMA to perform the comet assay.

Statistical analysis

The results are expressed as mean and standard deviation of at least three replicates obtained in independent experiments. In each replica of the comet assay a corresponding mean value of at least 50 comets was used to calculate the mean value of the 3 replicates. Statistical analysis was performed using analysis of variance (ANOVA) at the 1% significance level and the post-hoc Tukey test (p <0.05) to compare groups, including the fluorescence analysis test.

RESULTS

Analysis of DNA damage to *C. albicans* cells

To assess genotoxicity of PDT, cells were treated with 2.5 µM CUR and blue LED (37.5 J/cm^2; group P+L+) and the DNA damage was analyzed by the comet assay. Controls were included in the analysis: no treatment (P-L-), CUR only (P+L-), blue LED only (P-L+) and 10 mM H_2O_2 (group H_2O_2). Upon microscopic inspection of the comets after the treatments, as expected, group P-L- did not display considerable comet tails (Figure 1A) unlike group P+L+, which displayed longer comet tails (Figure 1B), indicating that PDT is genotoxic. The length of the comet tails was used as DNA damage parameter, longer tails being proportional to more abundant DNA damage. As depicted in Figure 1C, a statistically significant difference was observed between the group that received photodynamic therapy (P+L+) ($p \leq 0.0001$), the group that received only blue LED light (P-L+) ($p \leq 0.0001$), and the group treated with hydrogen peroxide (H_2O_2) ($p \leq 0.0001$). These groups displayed statistically significant differences from the negative control group (P-L). Interestingly, blue LED only was able to promote DNA damage (see P-L+ and P-L- in Figure 1C), which was significantly increased when cells were previously treated with CUR when irradiated in PDT (see P+L+ in

Figure 1C). In addition, the group treated with CUR (P+L-) was similar to the P-L- group, suggesting that this compound is not genotoxic ($p>0.993$).

Analysis of intracellular ROS production

Photosensitization is usually mediated by the production of ROS when the PS is excited by light. To investigate if the genotoxicity of PDT is provoked by ROS upon irradiation, cells were loaded with the redox sensitive fluorochrome H_2DCF-DA and then treated as in the previous experiment. The apolar nature of the fluorochrome allows free permeation through biological membranes, however, once inside cells, esterases deacetylate H_2DCF-DA yielding the polar compound dichlorofluorescein (H_2DCF), which becomes trapped inside the cells. Upon oxidation H_2DCF is converted into the fluorescence-emitting oxidized form. Therefore, fluorescence of H_2DCF-DA-loaded cells is proportional to the amount of ROS produced during the treatments. Figure 2 shows that the fluorescence emitted by cells treated only with light (P-L+) was significantly higher than that of the groups receiving PDT (P+L+) ($p\leq0.032$) or H_2O_2 ($p\leq0.028$), which were similar among them, suggesting that ROS production was more significant in group P-L+ ($p\leq0.0001$). These groups displayed a statistically significant increase of fluorescence when compared with the negative control (P-L-), while the group treated only with CUR (P+L-) showed a slight but non-significant increase in fluorescence when compared to group P-L- ($p>0.437$). Figure 3 shows the difference in the fluorescence of cells that have undergone oxidative stress of the groups P-L- and P-L+, which corroborated the intracellular origin of the fluorescence. In addition, further testing was conducted with the group treated with CUR (without H_2DCF-DA) to assess whether CUR alone would emit the same fluorescence spectrum as H_2DCF-DA. The results showed absence of fluorescence of the cells analyzed (data not shown).

ROS production induced by blue LED (P-L+; Figure 2) correlates with the genotoxicity observed in the previous experiment (see P-L+ in Figure 1C) and suggests that this radiation promotes DNA damage through the induction of production of ROS inside the cells. Nevertheless, considering that blue LED is able to induce the highest amount of ROS, DNA damage would be expected to be more extensive in this group than in the PDT group (P+L+; see Figures 1C and 2). One reason for this could be an inhibition of DNA damage repair by CUR that would exacerbate blue LED genotoxicity.

Analysis of DNA damage repair in *C. albicans* cells

To investigate if CUR is inhibiting DNA damage repair during PDT, cells were exposed to treatments as in the previous experiments and then they were incubated for different periods of time to allow the activity of the DNA damage repair systems, before DNA damage analysis by the comet assay. Figure 4 shows the DNA damage repair activity through the analysis of DNA damage along time for each group of cells. Except for untreated cells (P-L-) and cells treated only with CUR (P+L-), where the initial DNA damage was negligible, the size of comet tails decreased over time, suggesting that DNA damage is actively repaired during the experiment.

Repair was significantly more accentuated in cells in the P-L+ group in which the tail length decreased approximately 90% after 30 min when compared with time zero, which reached similar levels of DNA damage as in P-L-, the negative control group (Figures 4, 5A and 5B). The cells submitted to PDT (P+L+) showed a reduction in tail length of only approximately 45% after 30 min compared with time zero (Figures 4, 5C and 5D). These results strongly suggest that CUR inhibits DNA damage repair during PDT, which contributes to an enhanced genotoxic effect of blue LED. However, P+L+ cells still display more DNA damage than cells of group P-L+, indicating that other factor(s) might be contributing to enhance the genotoxic effect in PDT.

DISCUSSION

Photodynamic therapy (PDT) is considered a minimally invasive method for the treatment of infectious diseases such as OPC, which is mediated by the production of ROS that are responsible for the inactivation of target cells (Lyon *et al*., 2011). Some investigations have demonstrated that ROS are the main causative agent of DNA damage on fungi cells, and the level of this alteration can be easily detected by the comet assay (Henle *et al*., 1996; Azevedo *et al*., 2011). The comet assay has widespread applications allowing an estimation of the degree of genotoxicity of potentially mutagenic and/or carcinogenic agents in studies of DNA damage and repair (Speit & Hartmann, 1999; Speit *et al*., 1999). The present study was conducted to assess the DNA damage caused by the oxidative stress generated by CUR-mediated PDT associated with blue LED light on *C. albicans* and the inhibition of DNA repair provoked by CUR.

Oxidative stress is caused by ROS, such as, superoxide and hydroxyl radical, and more specifically singlet oxygen that pose a significant threat to cell integrity by causing damage to the DNA, lipids, proteins, and other cellular macromolecules (Slupphau *et al*., 2003). In the present study, it was found that PDT using CUR (2.5 μM which correspond to

0,9 mg/L) associated with blue LED at 37.5 J/cm^2 (group P+L+) induced DNA damage. These modifications were more significant when compared with the group treated with H_2O_2 (Figure 1C). These results may be justified by the fact that yeast cells are aerobic and produce ROS during their natural metabolism in the mitochondrial respiratory chain and in peroxisomal metabolism that generates H_2O_2 in reactions catalyzed by oxidases (Moradas-Ferreira *et al*., 1996). Endogenous ROS are dispersed from the mitochondrial electron transport chain and peroxisomes, diffusing freely through the cell membranes, attacking other components inside the cell (Salmon *et al*., 2004). Oxidative damage produced by endogenous ROS can induce DNA base modifications, single- and double-strand breaks, in addition to the formation of purine/pyrimidine lesions, many of which are liable to be toxic and/or mutagenic (Girard & Boiteux, 1997). Endogenous ROS production added to those produced by light associated with CUR may have been responsible for accentuating the genetic damage of the *C. albicans* cells.

After the application of blue LED light (group P-L+), DNA cell damage was also observed regardless of the use of a PS. Studies have reported that the wavelength used in the present study (455 nm) causes oxidative stress that may be associated with the photoexcitation of endogenous intracellular porphyrins in a way that the absorption of these photons would promote energy transfer to oxygen, culminating in the production of ROS (Hamblin *et al*., 2005; Maclean *et al*., 2008). It has been reported that microorganisms can detect blue light, which may induce physiological responses caused by the light receptors (Daia *et al*., 2012). Thus, photosensitized excitation of blue light chromophores, including cytochromes and flavins, within the mitochondria and/or peroxisomes could induce the production of endogenous ROS (Daia *et al*., 2012). The presence of comets in *C. albicans* cells was also observed after UV irradiation (352 nm) (Farrell *et al*., 2011). Furthermore, it has been reported that blue LED light has an antimicrobial effect on bacteria such as *Staphylococcus aureus* (Guffey & Wilborn, 2006), including methicillin-resistant strains (Enwemeka *et al*., 2008) and *Pseudomonas aeruginosa* (Guffey & Wilborn, 2006). According to studies that have evaluated the visible band spectrum, the wavelength range from 402 to 420 nm has been reported to be the most effective against microorganisms, while the spectrum from 455 to 470 nm showed antimicrobial potential only in some bacterial species (Daia *et al*., 2012). Studies have shown that the wavelength (455 nm) and light dose of 37.5 J/cm^2 used in the present study has no antifungal effect (Dovigo *et al*., 2011a; Dovigo *et al*., 2013). However, this investigation demonstrated that this wavelength was able to promote

DNA damage on *C. albicans*. It can be justified by the fact that *C. albicans* cells have the ability of efficiently repair the DNA damage under these conditions so that they can survive.

Fluorescence assessment with H_2DCF-DA revealed that PDT, light, and H_2O_2 provoked oxidative stress. The fluorescence intensity of the group treated only with light (P-L+) was more accentuated, suggesting that ROS production was higher in this group (Figure 2). These results may have occurred because the blue light stimulated intracellular ROS production, as discussed above. Interestingly, lower fluorescence was observed when cells were submitted to PDT. It has been reported that a high concentration of CUR alters the expression of genes that regulate the metabolism, and mitochondrial integrity of *C. albicans* (Kumar *et al*., 2014). Possibly, CUR may have acted in the mitochondria, impairing cellular respiration, resulting in lower endogenous ROS production. Although, a low concentration of CUR was used in the present study, we suggest that light might have enhanced the effect of CUR in the cell. Thus, it is possible to mention that low concentration of CUR associated with blue LED light altered the cellular metabolism, which can result in lower quantity of ROS produced in the mitochondria during the cell respiration. On the other hand the antioxidant activity of CUR (Barzegar & Moosavi-Movahedi, 2011) might have also contributed to lower fluorescence in PDT. Scavenging of light-induced ROS by CUR in PDT would contribute to attenuate the oxidative stress.

Furthermore, we evaluated the kinetics of DNA repair in fungal cells with the purpose of verifying if the damaged DNA would be repaired over time. The results showed that CUR when combined with blue LED light (PDT; P+L+) was genotoxic and hindered DNA repair of *C. albicans* cells, which was characterized by the reduction of the comet tails size in only approximately 45% after 30 min, unlike the group of cells exposed to blue LED without CUR (P-L+) where the reduction was 90% (Figures 4 and 5). The influence of CUR on DNA repair pathways in yeast cells is still not completely understood, according to the literature (Azad *et al*., 2013). Topoisomerases are the main enzymes responsible to directly alter topological changes in DNA and thereby playing an important role in the regulation of the replication, transcription, recombination and chromosome condensation (Corbett & Berger, 2004). The activities of this enzyme are crucial in cells with DNA damage (Champoux, 2001). To our knowledge, only one study reported the influence of CUR on the enzymatic activities of topoisomerases I and II (top I and II) of *S. cerevisiae* and *C. albicans* (Roth *et al*., 1998). According to the authors a low concentration of CUR (0,001mg/L) was able to promote the inhibition of the activity of *C. albicans* top I and II (Roth *et al*., 1998). Thus, it is possible to

suggest that CUR also may have interacted with enzymes such as topoisomerase in the present study, hindering DNA repair, which would explain the slower DNA repair in the P+L+ group in comparison with P-L+.

In summary, the comet assay can be considered an important tool for assessing the integrity of the genome of *C. albicans* cells in the study of the toxicity of natural compounds and therapies targeted against fungal DNA. Results from the present investigation suggest that PDT provoked extensive DNA damage that was not repaired efficiently due to the inhibition caused by CUR. This may be the main mechanism of the antifungal activity of CUR-mediated PDT. It is possible that virulence factors of this microorganism might also be affected upon PDT, which would exacerbate the therapeutic effect. Therefore it is important to investigate the effects of PDT also in *in vivo* studies by monitoring DNA damage and virulence factors. This will help to understand the mechanism of action of PDT on *C. albicans* cells that will allow implementing efficiently this therapeutic approach in the clinical practice.

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References

1. Andrade MC, Ribeiro APD, Dovigo LN, Brunetti IL, Giampaolo ET, Bagnato VS, Pavarina AC (2013) Effect of different pre-irradiation times on curcumin-mediated photodynamic therapy against planktonic cultures and biofilms of *Candida* spp. *Arch Oral Biol* **58**: 200-210.
2. Azad GK, Singh V, Golla U, Tomar RS (2013) Depletion of Cellular Iron by Curcumin Leads to Alteration in Histone Acetylation and Degradation of Sml1p in *Saccharomyces cerevisiae. PLoS One* **8**: e59003.
3. Azevedo F, Marques F, Fokt H, Oliveira R. Johansson B (2011) Measuring oxidative DNA damage and DNA repair using the yeast comet assay. *Yeast* **28**: 55–61.
4. Barzegar A, Moosavi-Movahedi AA (2011) Intracellular ROS protection efficiency and free radical-scavenging activity of curcumin. *PLoS One* **6**: e26012.

5. Bliss JM, Bigelow CE, Foster TH, Haidaris CG (2004) Susceptibility of *Candida* species to photodynamic effects of Photofrin. *Antimicrob Agents Chemother* **48**: 2000-2006.
6. Boiteux S, Guillet M (2004) A basic sites in DNA: repair and biological consequences in *Saccharomyces cerevisiae*. *DNA Repair (Amst)* **3**: 1-12.
7. Bonnett R, Martínez G (2001) Photobleaching of sensitisers used in photodynamic therapy. *Tetrahedron* **57**: 9513-9547.
8. Bruzell EM, Morisbak E, Tønnesen HH (2005) Studies on curcumin and curcuminoids. XXIX. Photoinduced cytotoxicity of curcumin in selected aqueous preparations. *Photochem Photobiol Sci* **4**: 523-530.
9. Cannon RD, Holmes AR, Mason AB, Monk BC (1995) Oral *Candida*: Clearance, colonization, or candidiasis? *J Dent Res* **74**: 1152-1161.
10. Champoux JJ (2001) DNA topoisomerases: structure, function, and mechanism. *Annu Rev Biochem* **70**: 369-413.
11. Collins AR (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol* **26**: 249-261.
12. Copley L, Vander WP, Wirtz KW, Parker MI, Leaner VD (2008) Photolon, a chlorin e6 derivative, triggers ROS production and light-dependent cell death via necrosis. *Int J Biochem Cell B* **40**: 227-235.
13. Corbett KD, Berger JM (2004) Structure, molecular mechanisms, and evolutionary relationships in DNA topoisomerases. *Annu Rev Bioph Biom* **33**: 95-118.
14. Daia T, Guptaa A, Murrayd CK, Vrahase MS, Tegos GP, Hamblin MR (2012) Blue light for infectious diseases: *Propionibacterium acnes, Helicobacter pylori,* and beyond? *Drug Resist Update* **15**: 223-236.
15. Demidova TN, Hamblin MR (2005) Effect of cell-photosensitizer binding and cell density on microbial photoinactivation. *Antimicrob Agents Chemother* **49**: 2329-2335.
16. Donnelly RF, McCarron PA, Tunney MM (2008) Antifungal photodynamic therapy. *Microbiol Res* **163**: 1-12.

17. Dovigo LN, Pavarina AC, Carmello JC, Machado AL, Brunetti IL, Bagnato VS (2011a). Susceptibility of clinical isolates of *Candida* to photodynamic effects of curcumin. *Laser Surg Med* **43**: 927-934.
18. Dovigo LN, Pavarina AC, de Oliveira Mima EG, Giampaolo ET, Vergani CE, Bagnato VS (2011b) Fungicidal effect of photodynamic therapy against fluconazole-resistant *Candida albicans* and *Candida glabrata*. *Mycoses* **54** : 123-130.
19. Dovigo LN, Pavarina AC, Ribeiro AP, Brunetti IL, Costa CA, Jacomassi DP, Bagnato VS, Kurachi C (2011c) Investigation of the photodynamic effects of curcumin against *Candida albicans*. *Photochem Photobiol* **87**: 895-903.
20. Dovigo LN, Carmello JC, de Souza Costa CA, Vergani CE, Brunetti IL, Bagnato VS, Pavarina AC (2013) Curcumin-mediated photodynamic inactivation of *Candida albicans* in a murine model of oral candidiasis. *Med Mycol* **51**: 243-251.
21. Enwemeka CS, Williams D, Hollosi S, Yens D, Enwemeka SK (2008) Visible 405 nm SLD light photo-destroys methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro*. *Laser Surg Med* **40**: 734-737.
22. Farrell H, Hayes J, Laffey J, Rowan N (2011) Studies on the relationship between pulsed UV light irradiation and the simultaneous occurrence of molecular and cellular damage in clinically-relevant *Candida albicans*. *J Microbiol Methods* **84**: 317-326.
23. Girard PM and Boiteux S (1997) Repair of oxidized DNA bases in the yeast *Saccharomyces cerevisiae*. *Biochimie* **79**: 559-566.
24. Gois MM, Kurachi C, Santana EJ, Mima EG, Spolidório DM, Pelino JE, Salvador Bagnato V (2010) Susceptibility of *Staphylococcus aureus* to porphyrin-mediated photodynamic antimicrobial chemotherapy: an *in vitro* study. *Laser Med Sci* **25**: 391-395.
25. Guffey JS, Wilborn J (2006) *In vitro* bactericidal effects of 405-nm and 470-nm blue light. *Photomed Laser Surg* **24**: 684-688.
26. Hamblin MR, Viveiros J, Yang C, Ahmadi A, Ganz RA, Tolkoff MJ (2005) *Helicobacter pylori* accumulates photoactive porphyrins and is killed by visible light. *Antimicrob Agents Chemother* **49**: 2822-2827.
27. Henderson BW, Dougherty TJ (1992) How does photodynamic therapy work? *Photochem Photobiol* **55**: 145-157.

28. Henle ES, Luo Y, Gassmann W, Linn S (1996) Oxidative damage to DNA constituents by iron-mediated fenton reactions. The deoxyguanosine family. *J Biol Chem* **271**: 21177-21186.

29. Jori G, Fabris C, Soncin M, Ferro S, Coppellotti O, Dei D, Fantetti L, Chiti G, Roncucci G (2006) Photodynamic therapy in the treatment of microbial infections: basic principles and perspective applications. *Laser Surg Med* **38**: 468-481.

30. Junqueira JC, da Silva Martins J, Faria RL, Colombo CE, Jorge AO (2009) Photodynamic therapy for the treatment of buccal candidiasis in rats. *Laser Med Sci* **24**: 877-84.

31. Konopka K, Goslinski T (2007) Photodynamic Therapy in Dentistry. *J Dent Res* **86**: 694-707.

32. Krcmery V, Barnes AJ (2002) Non-*albicans Candida* spp. causing fungaemia: Pathogenicity and antifungal resistance. *J Hosp Infect* **50**: 243-260.

33. Kumar A, Dhamgaye S, Maurya IK, Singh A, Sharma M, Prasad R (2014). Curcumin Targets Cell Wall Integrity via Calcineurin-Mediated Signaling in *Candida albicans*. *Antimicrob Agents Chemother* **58**: 167-175.

34. Lambrechts SAG, Aalders MCG, Van Marle J (2005) Mechanistic study of the photodynamic inactivation of *Candida albicans* by cationic porphyrin. *Antimicrob Agents Chemother* **49**: 2026-2034.

35. Lyon JP, Moreira LM, Moraes PCG, Santos FV, Resende MA (2011) Photodynamic therapy for pathogenic fungi. *Mycoses* **54**: 265-271.

36. Maclean M, Macgregor SJ, Anderson JG, Woolsey GA (2008) The role of oxygen in the visible-light inactivation of *Staphylococcus aureus*. *J Photoch Photobio B* **92**: 180-184.

37. Mantareva V, Kussovski V, Angelov I, Wo¨hrle D, Dimitrov R, Popova E, Dimitrov S (2011) Non-aggregated Ga(III)-phthalocyaninesin the photodynamic inactivation of planktonic and biofilm cultures of pathogenic microorganisms. *Photoch Photobio Sci* **10**: 91-102.

38. Moradas-Ferreira P, Costa V, Piper P, Mager W (1996) The molecular defences against reactive oxygen species in yeast. *Mol Microbiol* **19**: 651-658.

39. Paardekooper M, Van den Broek PJ, De Bruijne AW, Elferink JG, Dubbelman TM, Van Steveninck J (1992) Photodynamic treatment of yeast cells with the dye toluidine blue: All-ornone loss of plasma membrane barrier properties. *Biochim Biophys Acta* **1108**: 86-90.

40. Pappas PG, Kauffman CA, Andes D, et al. (2009) Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin Infect Dis* **48**: 503-535.

41. Perezous LF, Flaitz CM, Goldschmidt ME, Engelmeier RL (2005) Colonization of *Candida* species in denture wearers with emphasis on HIV infection: A literature review. *J Prosthet Dent* **93**: 288-293.

42. Pfaller MA, Diekema DJ (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* **20**: 133-163.

43. Rank J, Syberg K, Jensen K (2009) Comet assay on tetraploid yeast cells. *Mutat Res* **673**: 53-58.

44. Roth GN, Chandra A, Nair MG (1998) Novel Bioactivities of *Curcuma longa* Constituents. *J Nat Prod* **61**: 542-545.

45. Salmon TB, Evert BA, Song B, Doetsch PW (2004) Biological consequences of oxidative stress-induced DNA damage in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **32**: 3712-3723.

46. Samaranayake LP (1990) Oral candidosis: an old disease in new guises. *Dent Update* **17**: 36-38.

47. Scully C, El-Kabir M, Samaranayake LP (1994) *Candida* and oral candidosis: A review. *Crit Rev Oral Biol M* **5**:125-157.

48. Shapiro RS, Robbins N, Cowen LE (2011) Regulatory circuitry governing fungal development, drug resistance, and disease. *Microbiol Mol Biol Rev* **75**: 213-267.

49. Sharma RA, Gescher AJ, Steward WP (2005) Curcumin: the story so far. *Eur J Cancer* **41**: 1955-1968.

50. Slupphau G, Kavli B, Krokan HE (2003) The interacting pathways for prevention and repair of oxidative DNA damage. *Mutat Res* **531**: 231-251.

51. Sonis ST, Peterson RL, Edwards LJ, Lucey CA, Wang L, Mason L, Login G, Ymamkawa M, Moses G, Bouchard P, Hayes LL, Bedrosian C, Dorner AJ (2000)

Defining mechanisms of action of interleukin-11 on the progression of radiation-induced oral mucositis in hamsters. *Oral Oncol* **36**: 373-381.

52. Speit G, Hartmann A (1999) The comet assay (single-cell gel test) - sensitive genotoxicity test for the detection of DNA damage and repair. *Methods Mol Biol* **113:** 203-212.

53. Speit G, Trenz K, Schütz P, Rothfuß A, Merk O (1999) The influence of temperature during alkaline treatment and electrophoresis on results obtained with the comet assay. *Toxicol Lett* **110**: 73-78.

54. Strackhovskaya MG, Belenikina NS, Ivanova EV, Chemeris YK, Stranadko EF (2002) The photodynamic inactivation of the yeast *Candida guilliermondii* in the presence of photodithazine. *Microbiol* **71**: 298-301.

55. Wainwright M (2004) Photoinactivation of viruses. *Photoch Photobio Sci* **3**: 406-411.

56. Zeina B, GreenmanJ, Purcell WM, Das B (2001) Killing of cutaneous microbial species by photodynamic therapy. *Brit J Dermatol* **144**: 274-278.

Figure legends

Figure 1 (A) – Imaging by fluorescence microscopy, representative of the group P-L-, showing the absence of comets; and (B) group P+L+ showing the presence of comets in the *C. albicans* cells

Figure 1 C- Imaging by fluorescence microscopy of yeast comets, upon DNA staining with GelRed, representative of the group P-L- (A), showing the absence of comets; and group P+L+ (B) showing the presence of comets in *C. albicans* cells. Magnification: 400x. (C) Mean and standard deviation of the length of the tails formed by the exposure of the *C. albicans* cells to PDT (group P+L+), CUR (P+L-), light (P-L+), or 10 mM hydrogen peroxide (H_2O_2). Negative control (P-L-) refers to untreated cells. The same superscript letters on the bars denote statistical similarity among groups (post-hoc Tukey test - p <0.05).

Figure 2 - Mean value and standard deviation of ROS production, measured as fluorescence intensity upon oxidation of H2DCF-DA by fluorimetry, of *C. albicans* submitted to PDT (P+L+), CUR (P+L-), light alone (P-L+), or 10 mM hydrogen peroxide (H2O2) in comparison with the control group (P-L-) ($p<0.05$). *Group with untreated cells and without fluorochrome. A.U. - arbitrary units.

Figure 3 - Representative images of fluorescence intensity of H2DCF-DA-loaded *C. albicans* cells of groups P-L-, untreated (A), and P-L+, treated only with blue LED (B). Magnification: 20x.

Figure 4 - DNA damage, represented as mean comet tail lengths, along time after treatments in groups P+L+ (PDT), P+L- (treated with CUR), P-L+ (treated with blue LED), and P-L- (untreated).* Statistically different from P-L+ ($p<0.05$).

Figure 5 - Representative images of DNA damage, depicted as yeast comets, in the DNA damage repair experiments with group P-L+ (irradiation with blue LED only; A and B) and with group P+L+ (PDT; C and D). DNA damage was assessed immediately after light application (A and C) and after 30 min incubation (B and D)

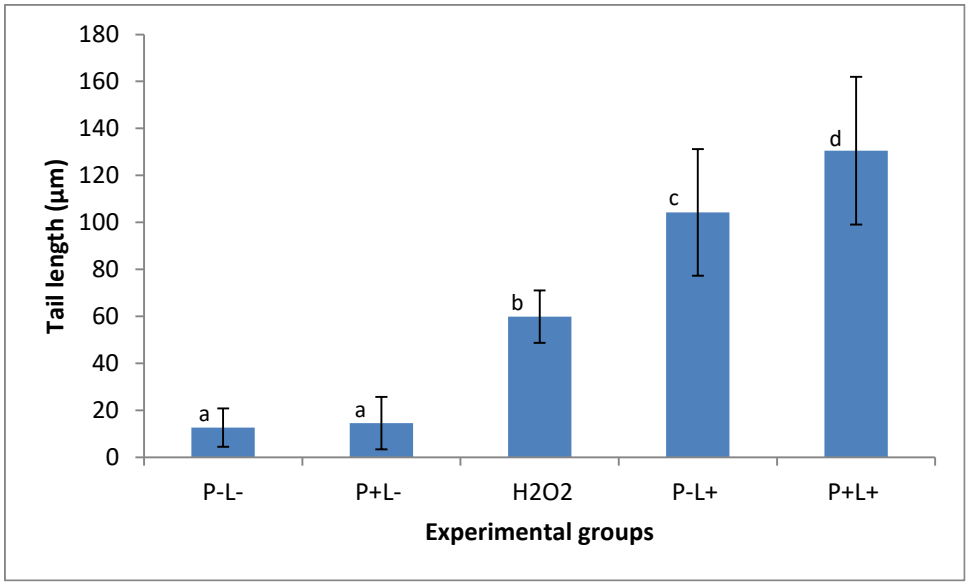

Figure 1 - Mean and standard deviation of the length of the tails (µm) formed by the exposure of the *C. albicans* cells to PDT (group P+L+), CUR (P+L-), light (P-L+), and H_2O_2. Negative control (P-L-) refers to untreated cells. The same superscript letters on the bars denote statistical similarity among groups. (Post-hoc Tukey test - $p < 0.05$)

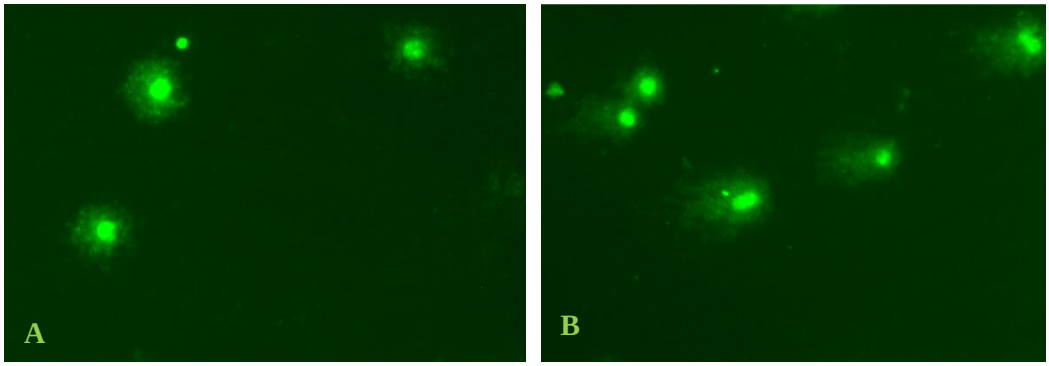

Figure 2 – Imaging by fluorescence microscopy, representative of the groups P+L- and P-L-, showing the absence of comets (A); and groups P+L+, P-L+, and H_2O_2, showing the presence of comets in the *C. albicans* cells.

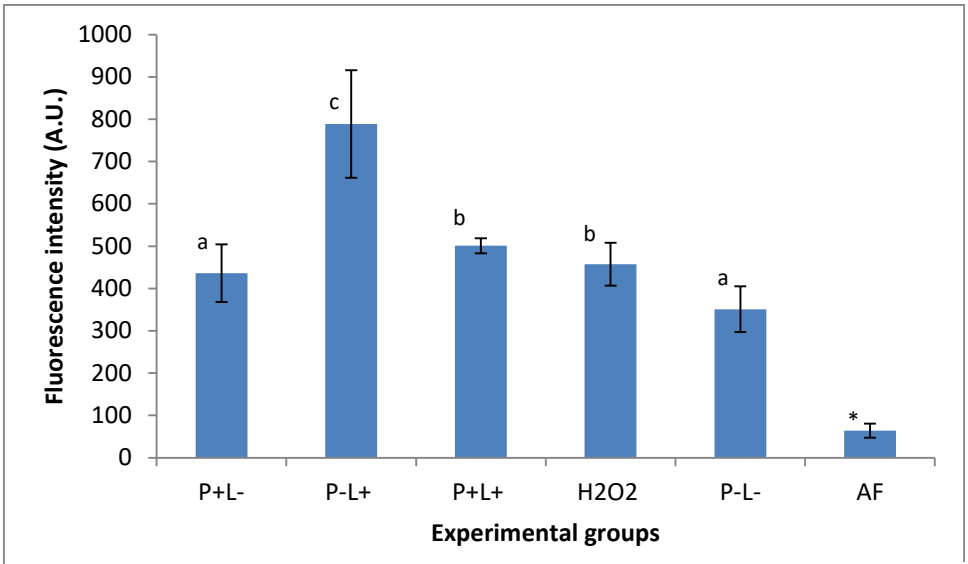

Figure 3 - Mean value and standard deviation of ROS production (fluorescence intensity) of *C. albicans* submitted to PDT (P+L+), CUR, light alone (P+L- and P-L+, respectively), or H_2O_2 in comparison with the control group (P-L-) ($p > 0.05$). *Group with untreated cells and without fluorochrome.

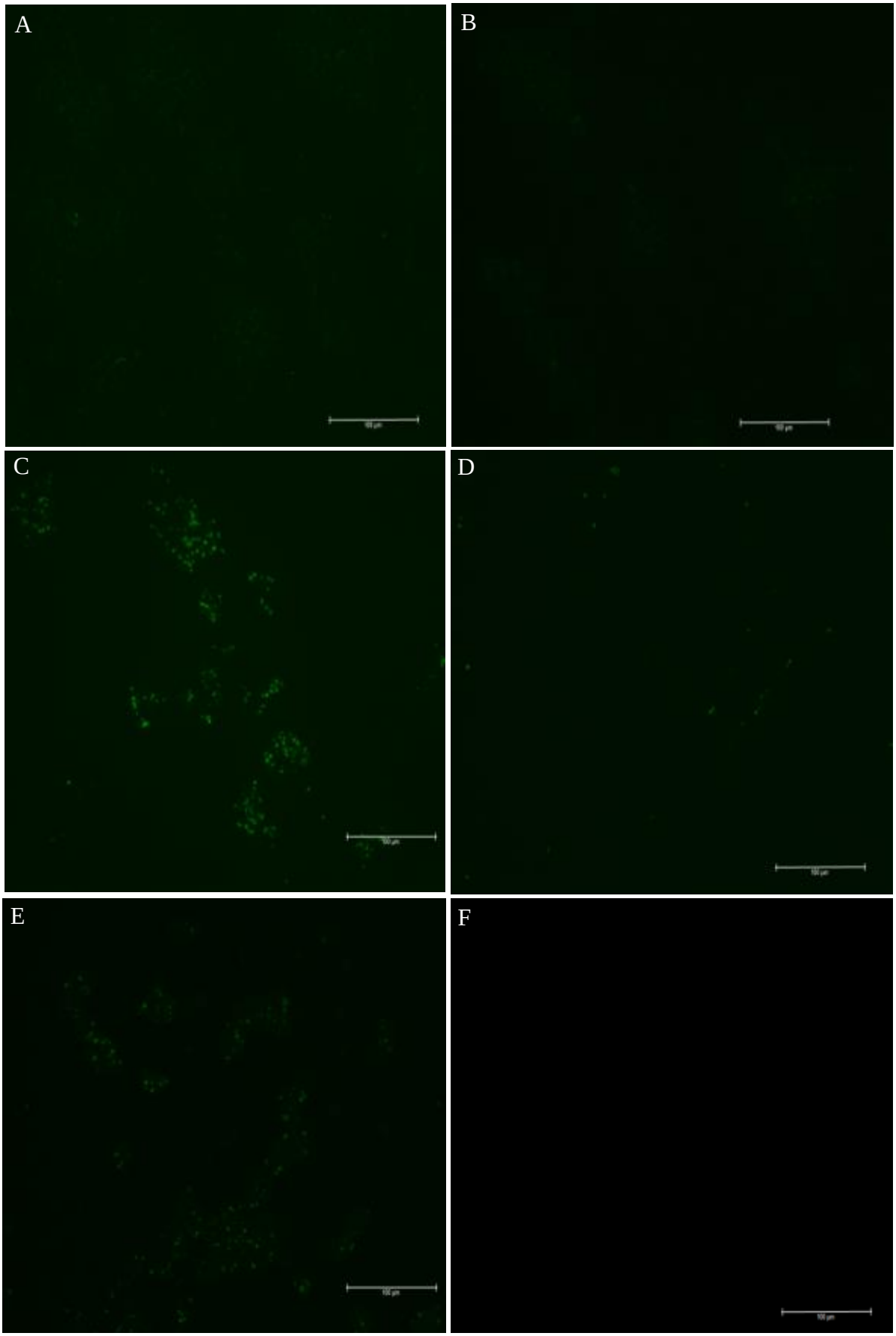

Figure 4 - Representative images of fluorescence intensity of the cells (20X) in all groups assessed: P-L- (A), P+L- (B), P-L+ (C), P+L+ (D), H_2O_2 (E), and AF (F).

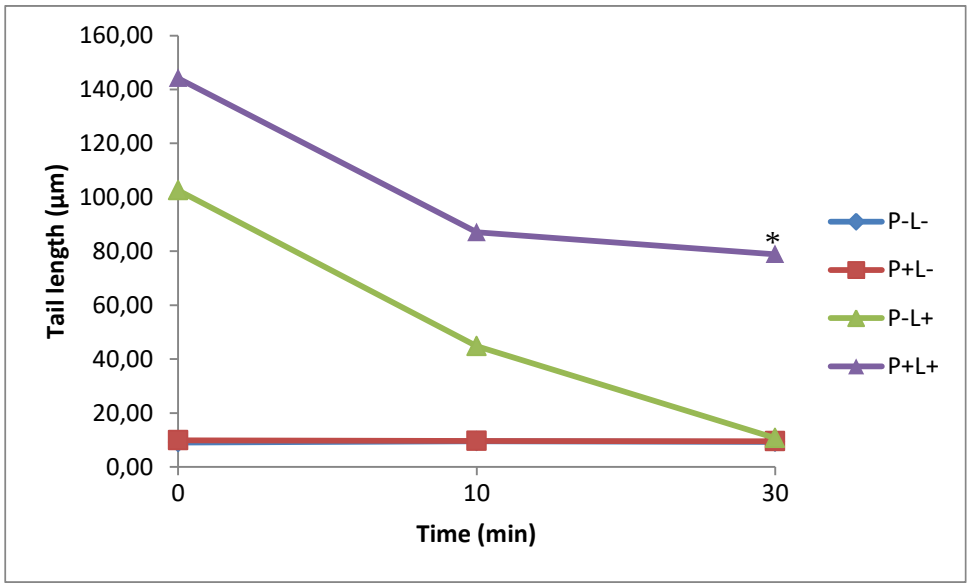

Figure 5 - Mean tail lengths (µm) in groups P+L+, P+L-, P-L+, and P-L- due to time. Assessment of DNA repair within 30 minutes after treatment.* Statistically different from the negative control group (P-L-) ($p>0.05$).