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Cytotoxic, biochemical and genotoxic effects of biodiesel produced by different routes on ZFL cell line

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ABSTRACT

Transesterification has proved to be the best option for obtaining biodiesel and, depending on the type of alcohol used in the reaction, the type of biodiesel may be methyl ester or ethyl ester. Leaking biodiesel can reach water bodies, contaminating aquatic organisms, particularly fish. The objective of this study was to determine whether the soluble fraction of biodiesel (Bd), produced by both the ethylic (BdEt) and methylic (BdMt) routes, can cause cytotoxic, biochemical and genotoxic alterations in the hepatocyte cell line of *Danio rerio* (ZFL). The metabolic activity of the cell was quantified by the MTT reduction method, while genotoxic damage was analyzed by the comet assay with the addition of specific endonucleases. The production of reactive oxygen species (ROS) and antioxidant/biotransformation enzymes activity also were determined. The results indicate that both Bd increased ROS production, glutathione S-transferase activity and the occurrence of DNA damage. BdMt showed higher cytotoxicity than BdEt, and also caused oxidative damage to the DNA. In general, both Bd appear to be stressors for the cells, causing cytotoxic, biochemical and genetic alterations in ZFL cells, but the type and intensity of the changes found appear to be dependent on the biodiesel production route.

1. Introduction

The best option to obtain biodiesel has proved to be transesterification, a relatively simple process that generates fuel whose properties resemble those of diesel oil (Ferrari et al., 2005). Biodiesel is a biofuel produced by the chemical reaction between vegetable oil or animal fat and alcohol, methanol or ethanol, which, in the presence of a catalyzer, such as sodium hydroxide, originates methyl or ethyl esters (Gerpen, 2005). This process involves reacting vegetable oil with alcohol to form esters and glycerol. Depending on the type of alcohol used in the reaction, biodiesel may be of the methyl ester (obtained by using methanol) or ethyl ester (ethanol) type. International experience indicates a tendency for transesterification using methanol (methylic route). An alternative route proposed in Brazil involves the use of ethanol (ethyl route) in the mix, but this technology still requires improvements in the production process on a commercial scale (Prates et al., 2007). Methanol is more commonly used considering its physical and chemical properties (short chain and polarity). However, ethanol is becoming increasingly popular because it is renewable and far less toxic than methanol (Lima, 2004). Although biodiesel is considered an environmentally friendly fuel, few studies have investigated its potential impact on ecosystems, such as aquatic environments. In view of the increasing advances in the biodiesel industry, it is of paramount importance to assess the environmental hazards of this biofuel in order to prevent deleterious impacts on living beings (Leme et al., 2011).

Cells represent a key level of biological organization for the detection and understanding of common mechanisms of toxicity (Castaño et al., 2003). The interactions of anthropogenic chemical substances with the biota occur initially at the cellular level; hence, cellular responses not only are the first manifestations of toxicity but can also be used as appropriate tools for the early and sensitive detection of exposure to chemical substances (Fent, 2001). Cell viability analysis has been used in the field of ecotoxicology to evaluate the toxic effects caused by environmental pollutants (Bopp and Lettieri, 2008). Fish cell lines play an important role in toxicological research, serving as a model to study molecular mechanisms of toxicity and also as a test system for studying

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and monitoring the toxic effects of environmental contaminants (Bols et al., 2005). Among the main tools currently in use in fish cell lines to assess the toxicity of anthropogenic substances are cell viability assays such as the MTT reduction method (Castaño et al., 2003). The genotoxicity of substances in the aquatic environment is also being monitored in fish cell lines. The comet assay, which detects breaks in DNA strands, has received considerable attention in this evaluation because it is a fast and cheap method (Bols et al., 2005). On the other hand, the literature contains few investigations into changes in the oxidative parameters of fish cell lines, such as production of reactive oxygen species (ROS) and the activity of antioxidant and biotransformation enzymes. It is important to assess oxidative parameters in cells because they indicate whether the mechanism of action of a particular contaminant involves the production of ROS, which increases the possible occurrence of oxidative stress if the ROS are not neutralized and may damage all the types of biological molecules of cells, including DNA (Lushchak, 2011).

Considering that biodiesel production is growing as well as the possibility of this fuel reaching groundwater or water bodies, thereby contaminating the environment and aquatic organisms, especially fish, it is crucial that the toxicity of this biofuel be understood. The vast majority of studies in the literature that link aquatic species with biodiesel contamination focus on the analysis of biodiesel toxicity based on the lethal concentration, LC_{50} (Hollebone et al., 2007; Khan et al., 2007; Leite et al., 2011). Studies using biochemical and genetic biomarkers to evaluate the effects of biodiesel on aquatic organisms are still incipient (Nogueira et al., 2011). Therefore, this study aimed to determine whether biodiesel produced by both the methylic and ethylic routes can cause cytotoxic, biochemical and genotoxic alterations in the hepatocyte cell line of *Danio rerio* (ZFL), and to compare these alterations to determine if they are similar, regardless of the biodiesel production route.

2. Materials and methods

2.1. Cell line

The cell line used was ZFL, a liver cell line of *D. rerio* fish. The hepatocyte cell line (ZFL) was cultured in 10 mL of Leibovitz/RPMI medium supplemented with 10% fetal bovine serum in 25 cm^2 flasks, and kept in an incubator without CO_2 at 28 °C.

2.2. Biodiesel under study and preparation of the soluble fraction of biodiesel (Bd)

For the toxicity assays, samples of biodiesel were donated by the Paraná Institute of Technology (TECPAR). Two different types of biodiesel were used, both extracted from sunflower oil; however, one was produced by methanol transesterification (methylic route) and the other by ethanol transesterification (ethylic route). The biodiesels used in this study meet the quality standards established by the National Petroleum Agency (ANP).

The soluble fractions of biodiesel (Bd), produced both by the ethylic (BdEt) and methylic routes (BdMt), were prepared separately using the same methodology. To prepare the Bd, one part of biodiesel was mixed with one part of distilled water (1:1) and the mixture was stirred for 24 h. The upper insoluble fraction was discarded and the water-soluble fraction was collected and stored in opaque containers in a cold chamber for a maximum of five days, until the time of the experiments. For the experiments, the Bd was diluted in various proportions.

2.3. Exposure protocols

For the MTT assay, ZFL cells were seeded on a transparent 24-well plate at a density of 1.2×10^5 cells per well. The cells were incubated for 24 h solely with culture medium (CTR) or with different dilutions of BdEt or BdMt (5%, 10%, 20%, 40%, 60%, 80% and 100%), always with the same concentration of culture medium. A positive control using methyl methanesulfonate (MMS) was also prepared in a concentration of 1 mM.

For reactive oxygen species (ROS) assay, the ZFL cells were seeded at a density of 9×10^5 cells per well on a black 96-well plate. In order to measure the antioxidant and biotransformation enzymes activity, ZFL cells (9×10^6) were cultured in 75 cm^2 flasks, always in monolayers, in Leibovitz/RPMI medium supplemented with 10% fetal bovine serum. For the alkaline version of the comet assay, with the addition of specific endonucleases, the ZFL cells were seeded at a density of 10^6 cells in 25 cm^2 flasks. A positive control was also prepared with a concentration of 0.5 mM of MMS. The cells were treated only with the culture medium (CTR) or with the different dilutions of BdEt or BdMt (5%, 10% and 20%) into culture medium for 1, 3, 6 and 12 h. Following all exposure periods cell viability was checked by trypan blue exclusion test and the results showed cell viability above 90% for all tested dilutions (5%, 10% and 20%) of both Bd (data not shown).

2.4. MTT assay

The cytotoxic potential of both types of Bd was evaluated through the MTT reduction method according to Mosmann (1983). After 24 h exposure, 5 mg of MTT was added and the cells were incubated again for another 4 h. The culture medium was then removed, 200 μL of dimethylsulfoxide (DMSO) was added, and the absorbance corresponding to each sample was determined at 540 nm in a microplate reader. The absorbance obtained for the CTR cells was considered as 100% of cell viability (CV). The CV of the other samples was determined by the following formula: $CVK = [(AK - AB)/(ACTR - AB)] \times 100$ where: CVK = cell viability of the cells exposed to Bd; AK = absorbance found for cells exposed to Bd; ACTR = absorbance found for the control cells; AB = absorbance found for the blank (well containing only culture medium).

2.5. Generation of reactive oxygen species (ROS)

After the exposure periods, the medium was discarded and the reaction buffer (100 μL) containing 30 mM HEPES (pH 7.2), 200 mM KCl and 1 mM $MgCl_2$ was added to the samples in all the wells. The microplate was then placed in a spectrofluorometer programmed to operate at 28 °C. This procedure yielded the spontaneous fluorescence of each sample at excitation and emission wavelengths of 488 and 525 nm, respectively. After reading the microplate, the fluorescent compound 2,7dichlorofluorescein diacetate (H_2DCFDA) was immediately added to the wells at a final concentration of 40 μM. The microplate was analyzed again and the fluorescent compound DCF was detected (ex: 488 nm; em: 525 nm). ROS production was monitored for 30 min, with readings taken at 5-min intervals, and calculated based on the ratio of the fluorescence units (FU) over time, after adjusting the FU data to a second degree polynomial function, and results were expressed as the area of FU × min (Ferreira-Cravo et al., 2007).

2.6. Antioxidant and biotransformation enzyme activity

At the end of exposure periods the medium was discarded, the cells were washed twice with PBS (126.6 mM NaCl; 4.8 mM KCL; 1.5 mM $CaCl_2$; 3.7 mM $NaHCO_3$; 8.9 mM Na_2HPO_4; and 2.9 mM

NaH_2PO_4) and released from the flasks' walls with trypsin (0.125%). The samples were transferred to microtubes and centrifuged for 10 min at 10,640*g*. The culture medium was removed and 500 μL of PBS was added to resuspend the pellet. To disrupt the cells, the samples were sonicated 3 times for 5 min, alternating with 3-min pauses. To ensure cell disruption, an aliquot of each sample was placed in a Neubauer chamber and examined under a light microscope at 400× magnification. Cells were considered disrupted when no cells were visible in the Neubauer chamber. The samples were then centrifuged for 10 min at 10,640*g* and the supernatant removed and stored at −80 °C until the biochemical analyses were performed. For the biochemical analyses, 20 μL of sample was used in each assay. The activity of the biotransformation enzyme glutathione S-transferase (GST) was determined based on the complexation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB), using a spectrophotometer at 340 nm, according to Keen et al. (1976). The superoxide dismutase (SOD) activity was determined according to McCord and Fridovich (1969), by the inhibition of the reduction rate of cytochrome c by the superoxide radical, which was followed spectrophotometrically at 550 nm and 25 °C. Catalase (CAT) activity was determined according to Beutler (1975), by monitoring the H_2O_2 decomposition rate based on the decrease in absorbance at 240 nm. Glutathione peroxidase (GPx) activity was determined spectrophotometrically at 25 °C by the oxidation of $NADPH^+H^+$ in the presence of peroxide, according to Hopkins and Tudhope (1973), at 340 nm. Protein concentration was determined according to Bradford (1976) at 595 nm.

2.7. Standard comet assay with the addition of repair enzymes

The levels of DNA damage in cells exposed to both Bd were monitored by the standard comet assay, which detects only DNA strand breaks and alkali-labile sites. To increase the sensitivity of the test, an additional step, with the inclusion of repair enzymes after the lysis step was carried out. The repair enzymes used in this study were formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (Endo III), which convert respectively oxidized purines and pyrimidines into DNA fragments (Azqueta et al., 2011).

After the exposure periods, the medium was discarded and the flasks were then washed with PBS, trypsin and medium with fetal bovine serum. The content was homogenized, centrifuged and the supernatant was discarded, the pellet was then used for the preparation of slides for the comet assay, following the protocol described by Singh et al. (1988) and Collins (2000), with slight modifications. Aliquots of each of the samples were added to low-melting-point agarose (0.5%) and divided into six glass slides previously coated with a layer of normal-melting-point agarose (1.5%), covered with coverslips, and kept for 30 min at 4 °C for agarose solidification. The coverslips were then removed and a second layer of normal-melting-point agarose (1.5%) was added to the slides, the coverslips were returned, and the slides were kept for more 30 min at 4 °C. Finally, the coverslips were removed and the slides were placed in freshly prepared cold lysis buffer (2.5 M NaCl; 100 mM EDTA; 10 mM Tris, pH 10; 1% Triton X-100 and 10% DMSO).

After lysis the slides were washed with PBS and placed in Flare buffer (40 mM Hepes, 0.1 M KCl, 0.2 mg mL^{-1} BSA and 0.5 mM EDTA, pH 8) for 15 min. After this, six slides prepared from the same culture flask were placed in a wet chamber, and the following additions were made on them: two slides received 100 μL of the enzymes reaction buffer (10× Flare, and distilled H_2O and BSA), two others received 100 μL of endo III (1:100 dilution), and the last two received 100 μL of FPG (1:100 dilution). All the slides were covered with coverslips and incubated for 45 min at 37 °C, after that they were kept at 4 °C for 15 min, and then transferred to an electrophoresis chamber filled with freshly prepared cold alkaline buffer (1 mM EDTA and 300 mM NaOH, pH > 13). After 30 min the electrophoresis was performed at 25 V and 300 mA for 20 min and then the slides were kept for 15 min in a neutralizing solution (0.4 M Tris, pH 7.5), fixed with absolute ethanol and dried at room temperature. Immediately prior to analysis, the slides were stained with GelRed solution. The analysis was performed in a fluorescence microscope under 40× objective lens magnification, using a 510–560 nm excitation filter and a 590 nm barrier filter, and 100 nucleoids were counted per slide.

DNA damage was visually classified in four classes, according to the migration of DNA fragments, as follows: class 0 – tailless nucleoid surrounded by a few fragments; class 1 – a small tail smaller than the diameter of the nucleoid, class 2 – tail length 1–2 times the diameter of the nucleoid; class 3 – a tail length greater than twice the diameter of the nucleoid. The score for each treatment was determined by multiplying the number of nucleoids observed in each damage class by the value of the class (0, 1, 2 or 3).

The standard comet assay was used to assess nonspecific damage in the DNA molecule through the comparison between the mean comet scores of CTR × Bd (methylic or ethylic) × PC obtained from the slides exposed only to the reaction buffer. In order to verify oxidative damage to purines and pyrimidines bases, the mean standard comet scores were compared to the comet scores of the slides exposed to FPG and Endo III, respectively.

2.8. Statistical analysis

The results (CTR × dilutions of Bd (methylic or ethylic) × PC when applicable) were compared by parametric analysis of variance (ANOVA) or the Kruskal–Wallis nonparametric test, according to the distribution of the data (normality and homogeneity of variance). When necessary, differences were identified by the Student–Newman–Keuls (SNK) multiple comparison test. Values of $P < 0.05$ were considered significant and the results were expressed as mean ± standard error (SE) of at least four independent experiments.

3. Results

3.1. Cell viability

The results indicated that cell viability, based on MTT reduction, of ZFL cells exposed to 5% and 10% dilutions of both BdMt and BdEt (Fig. 1) showed no statistical difference when compared with cell viability of cells not exposed to the soluble fractions (CTR). On the other hand, an increase in cell viability was detected at 20% and 40% dilutions of both soluble fractions. At 60% dilution, BdMt proved to be totally cytotoxic (0% viability), while at this same dilution, the cells exposed to BdEt showed a 30% reduction in cell viability based on metabolic activity. At dilutions of 80–100% of

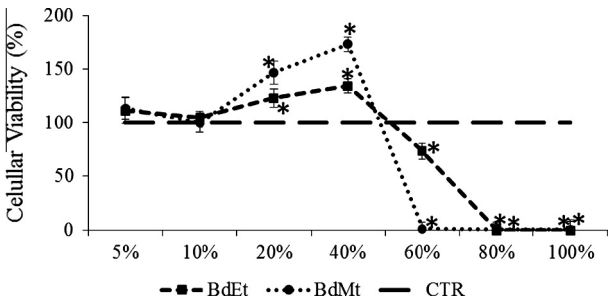

Fig. 1. Cell viability (%) of ZFL cells exposed to different dilutions of BdMt or BdEt, or only to culture medium (CTR) for 24 h. Results are mean ± SE of at least four independent experiments. *Indicates significant difference compared to the CTR, which was considered 100% ($P < 0.05$).

both soluble fractions the cell viability was reduced to 0%. MMS (CP) was completely cytotoxic (0% viability) to ZFL cells when compared with CTR (data not shown).

3.2. ROS generation

The results showed an increase in ROS production in cells exposed to 5%, 10% and 20% of BdMt for 6 h and to 10% and 20% of BdMt for 12 h, when compared to their respective negative control (Fig. 2A). Cells exposed for 6 h to 10% and 20% of BdEt also showed an increase in ROS production, as did cells exposed for 12 h to dilutions of 5%, 10% and 20%, both when compared to their respective negative control (Fig. 2B).

3.3. Antioxidant and biotransformation enzymes

GST activity showed a statistically significant increase in cells exposed to 5%, 10% and 20% of BdMt for 3 h and 6 h, and to 5–20% after 12 h of exposure when compared with their respective negative controls (Fig. 3A). GST activity was augmented only in cells exposed to 10% and 20% of BdEt for 12 h when compared to their respective negative controls (Fig. 3B). The SOD activity of cells exposed to both Bd did not differ statistically from respective negative controls at any exposure time to any dilution of the two soluble fractions (Fig. 4A and B). After 6 h exposure to 10% and 20% of BdMt, catalase activity was significantly augmented, but decreased significantly at the same dilutions after 12 h of exposure when compared to respective negative controls (Fig. 5A). In response to BdEt exposure, CAT activity did not differ statistically from respective negative controls at any dilution or exposure time (Fig. 5B). GPx activity showed no significant change in comparison to respective negative controls at any time or any dilution after exposure to the two soluble fractions of biodiesel (Fig. 6A and B).

3.4. DNA damage

In the standard comet assay a significant increase in DNA damage, in comparison to respective CTR, was detected in cells exposed

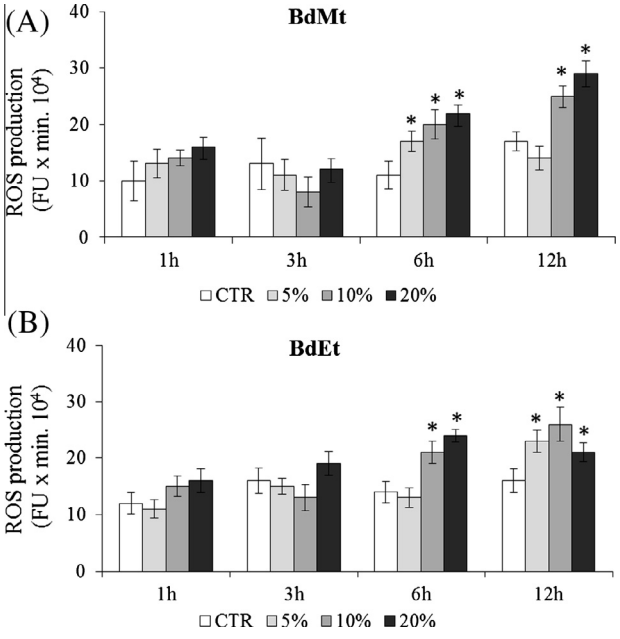

Fig. 2. Production of ROS in ZFL cells exposed to 5%, 10% and 20% of BdMt (A) and BdEt (B) or only to the culture medium (CTR) for 1, 3, 6 and 12 h. Results are mean ± SE of at least four independent experiments. *Indicates significant difference compared to the respective CTR ($P < 0.05$).

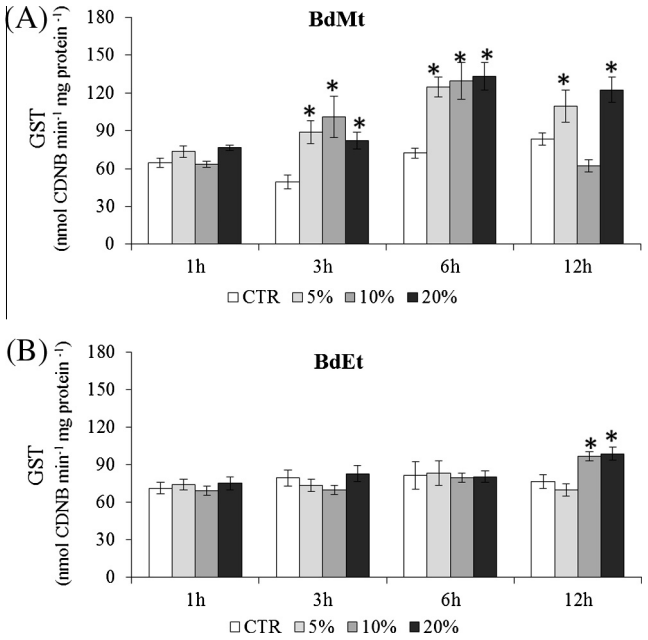

Fig. 3. GST activity in ZFL cells exposed to 5%, 10% and 20% of BdMt (A) and BdEt (B) or only to the culture medium (CTR) for 1, 3, 6 and 12 h. Results are mean ± SE of at least four independent experiments. *Indicates significant difference compared to the respective CTR ($P < 0.05$).

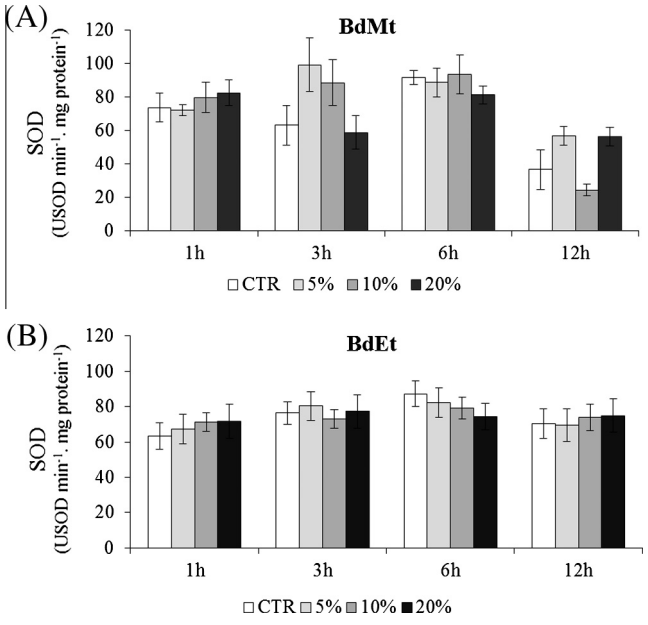

Fig. 4. SOD activity in ZFL cells exposed to 5%, 10% and 20% of BdMt (A) and BdEt (B) or only to the culture medium (CTR) for 1, 3, 6 and 12 h. Results are mean ± SE of at least four independent experiments.

to 10% and 20% of BdMt after 1 h, to 20% of BdMt after 3 h, and to 5%, 10% and 20% of BdMt after 6 h and 12 h, (Fig. 7A). BdEt caused a significant increase in DNA damage at dilutions of 5–20% within 1 h, at dilutions of 10% and 20% after 3 h, and at dilutions of 5%, 10% and 20% in 6 h and 12 h, compared with their respective negative controls (Fig. 7B). MMS (CP) was genotoxic at all exposure times, showing a significantly higher damage score than the negative CTR.

The comet assay using restriction enzymes on cells exposed to BdMt showed no statistically significant increase in DNA damage

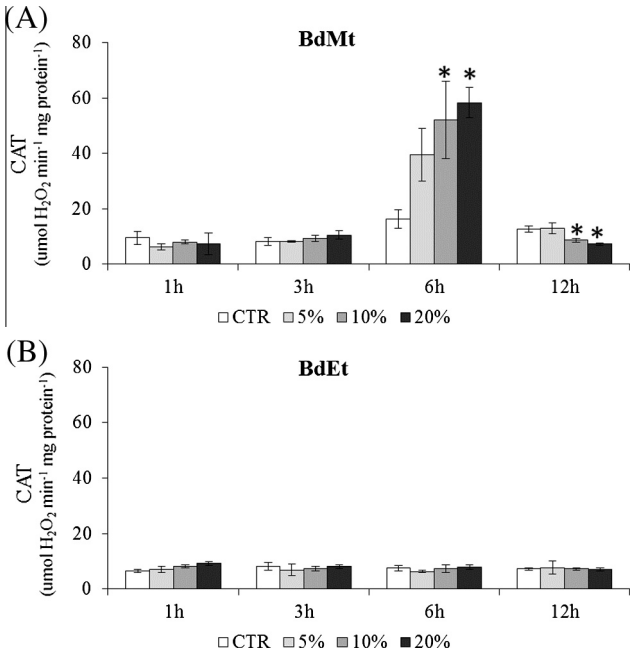

Fig. 5. CAT activity in ZFL cells exposed to 5%, 10% and 20% of BdMt (A) and BdEt (B) or only to the culture medium (CTR) for 1, 3, 6 and 12 h. Results are mean ± SE of at least four independent experiments. *Indicates significant difference compared to the respective CTR ($P < 0.05$).

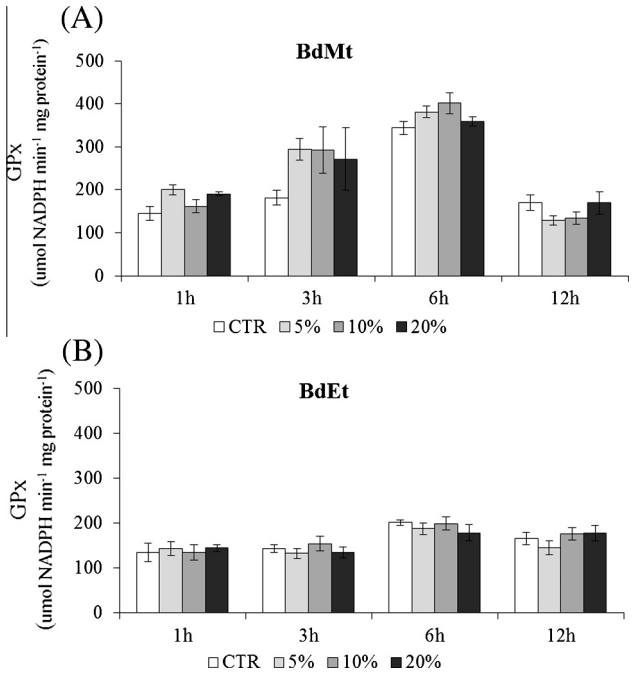

Fig. 6. GPx activity in ZFL cells exposed to 5%, 10% and 20% of BdMt (A) and BdEt (B) or only to the culture medium (CTR) for 1, 3, 6 and 12 h. Results are mean ± SE of at least four independent experiments.

of nucleoids treated with ENDO at any dilution and any time when compared to the nucleoids exposed only to the reaction buffer, under the same experimental conditions (Fig. 8A). On the other hand, the nucleoids treated with FPG showed a statistically significant increase at the dilution of 20% in 6 and 12 h when compared with nucleoids exposed only to reaction buffer under the same experimental conditions, indicating damage to purine bases (Fig. 8B). The comet assay using restriction enzymes on cells

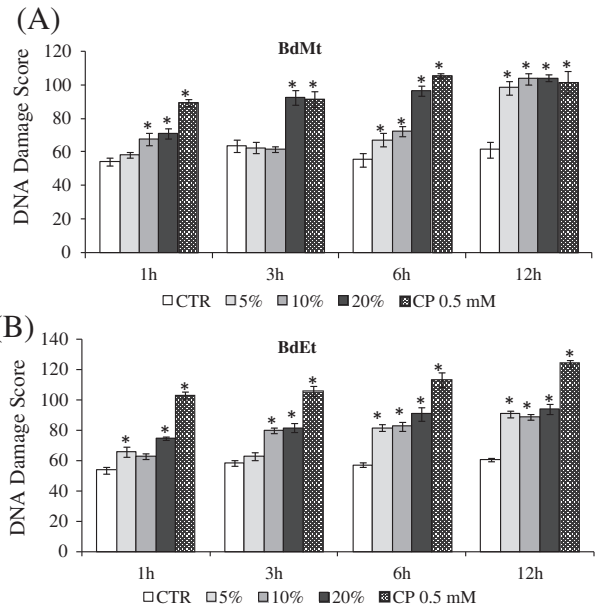

Fig. 7. Score of DNA damage in ZFL cells exposed to 5%, 10% and 20% of BdMt (A) and BdEt (B) or only to the culture medium (CTR) for 1, 3, 6 and 12 h, quantified by the standard comet assay. Results are mean ± SE of at least four independent experiments. *Indicates significant difference compared to the respective CTR ($P < 0.05$).

exposed to BdEt showed no statistical difference at any dilution or exposure time when compared to the nucleoids exposed only to reaction buffer under the same experimental conditions, indicating no oxidation of purine or pyrimidine bases (Fig. 9A and B).

4. Discussion

The MTT assay is a test that evaluates cell viability indirectly, by quantifying the cellular metabolic activity. MTT reduction is associated not only with the mitochondrial enzyme succinate dehydrogenase but also with NADP-dependent oxidoreductase enzymes located in the cytoplasm and non-mitochondrial membranes, including lysosome and plasma membranes (Berridge et al., 2005). This assay has been successfully used in fish cell lines, including ZFL (Seok et al., 2007; Bopp and Lettieri, 2008).

In this study, the results of the MTT assay indicated that the ZFL cells exposed to both methylic and ethylic Bd at dilutions of 5% and 10% remained viable, as did the cells exposed only to culture medium. In contrast, cells exposed to dilutions of 20% and 40% of both methylic and ethylic biodiesel showed an increase in metabolic activity when compared to non-exposed cells. At a dilution of 20% of methylic and ethylic Bd, the metabolic activity increased by 47% and 23%, respectively, while at 40% dilution, the metabolic activity increased by 73% and 35%, respectively. One of the possible explanations for these increases is that the Bd induces cell proliferation, although this hypothesis was not confirmed by counting cells in the Neubauer chamber (data not shown here). Another possible explanation is that the Bd may somehow alter the mitochondrial metabolism of cells, causing a higher reduction of tetrazolium into formazan. Huang and Huang (2011) found that the metabolism of ZFL cells was changed due to the overexpression of a subunit of succinate dehydrogenase in ZFL cells exposed to methyl parathion, and postulated that this increase was a compensatory mechanism to meet the decreased energy and biosynthesis after treatment with methyl parathion. In response to exposure to 60% or more of BdMt, cell viability began to decline and the Bd proved to be completely cytotoxic. However, exposure to 60% of BdEt was

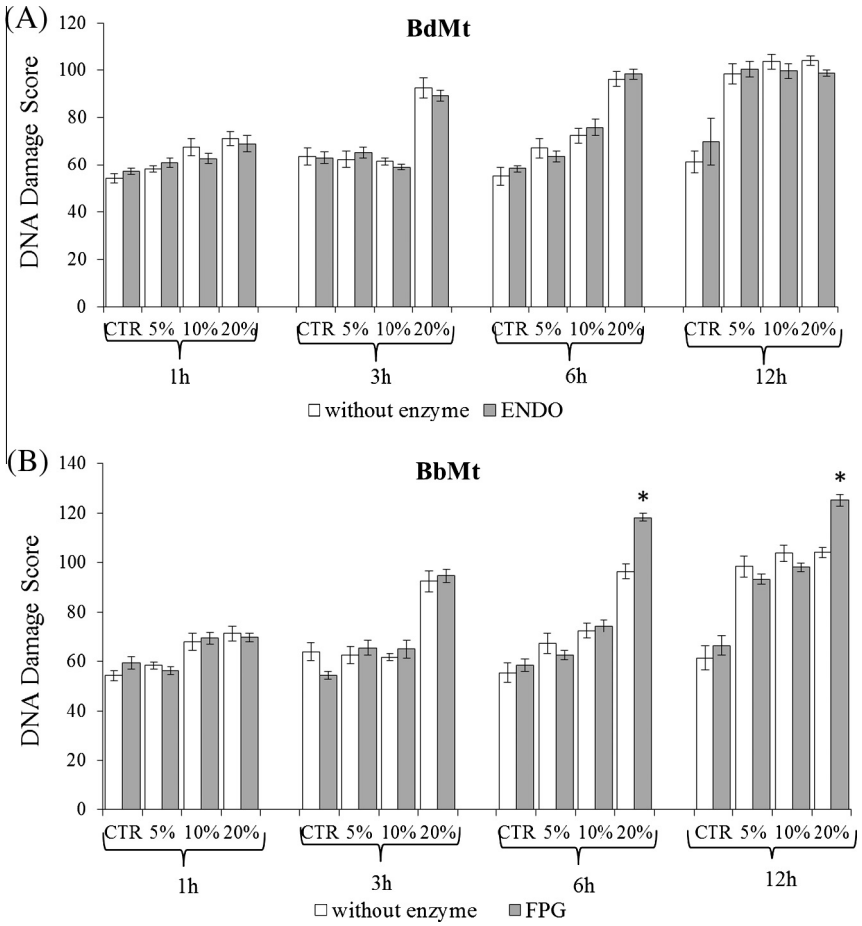

Fig. 8. Score of DNA damage in ZFL cells exposed to 5%, 10% and 20% of BdMt for 1, 3, 6 and 12 h, quantified by the standard comet assay (without enzyme) and the comet assay with the use of the enzyme endonuclease III (A) and FPG (B). Results are mean ± SE of at least four independent experiments. *Indicates significant difference from the respective condition without enzyme ($P < 0.05$).

not totally cytotoxic, showing a 30% loss of cell viability. In the case of BdEt, 100% cytotoxicity was observed only at dilutions of 80% or higher.

The cytotoxicity of Bd is presumably related with soluble compounds originating from biodiesel, such as dispersed droplets of fatty acid esters, alcohol residues (ethanol or methanol, depending on the production route), or due to the presence of elements from the raw material, the production process, or substances formed during the storage of biodiesel (De Quadros et al., 2011). The results of this study indicate that BdMt reduces 100% of the metabolic activity of cells at a lower dilution than BdEt. This may be related to the type of alcohol used in the production of each biodiesel, given that the toxicity of methanol is higher than that of ethanol, and this may have influenced the cytotoxic potential of the soluble fractions employed in this test.

An analysis of the kinetics of the MTT assay with cells exposed to the different dilutions of Bd indicates that the lower dilutions (5% and 10%) did not cause drastic changes in cell metabolism. As the dilution increased (20% and 40%), the cells responded by increasing their metabolic activity and thus remained alive. However, at high dilutions, the cells exposed to both soluble fractions did not survive (or were metabolically inactive). This type of curve, which is currently described in a number of toxicological studies by the name of hormesis, shows a biphasic dose response curve (Conolly and Lutz, 2004). The mechanisms that lead to the occurrence of hormesis are not clear. Zhang et al. (2008) hypothesized that adaptive responses may lead to hormesis. According to these authors, environmental stressors usually disturb the stable intracellular environment, but biological systems have a set of homeostatic control systems, at both the cellular and physiological level, that are activated to compensate for perturbations, adapting organisms to stressful environments. However, this cellular adaptation only occurs while the level of induction of anti-stress genes is not saturated. At low doses of exposure to the stressor, strong homeostatic control is expected, with coordinated changes in gene expression, keeping the cellular functions very similar to those of undisturbed cells. With intermediate level exposure, the system is less able to maintain homeostasis, because the anti-stress genes are approaching maximum induction. At high stressor levels, the cell's capacity to cope with the stressor is overcome, leading to cell death (Zhang et al., 2008). Descriptions have been made of the hormetic effect related to cell viability in fish cell lines such as 1-PLHC exposed to cadmium (Ryan and Hightower, 1994), RTG-2 exposed to zinc salts (Shúilleabháin et al., 2004), RTG-2 exposed to phenolic compounds (Davoren and Fogarty, 2006), and GCF exposed to copper (Tan et al., 2008).

In addition to the basal production of ROS, which the body produces by various pathways, exogenous substances (xenobiotics) can act on the generation of ROS. The most well known pollutants that act on this production are metals, aromatic hydrocarbons, pesticides, polychlorinated biphenyls, dioxins and many others (Lushchak, 2011). It is possible to infer that Bd, whether of methylic or ethylic origin, is a stressor that alters the production of ROS, leading to augmented levels of ROS within the cell. The increase may be related to the biotransformation of compounds of the soluble fractions, since the GST enzyme activity increased

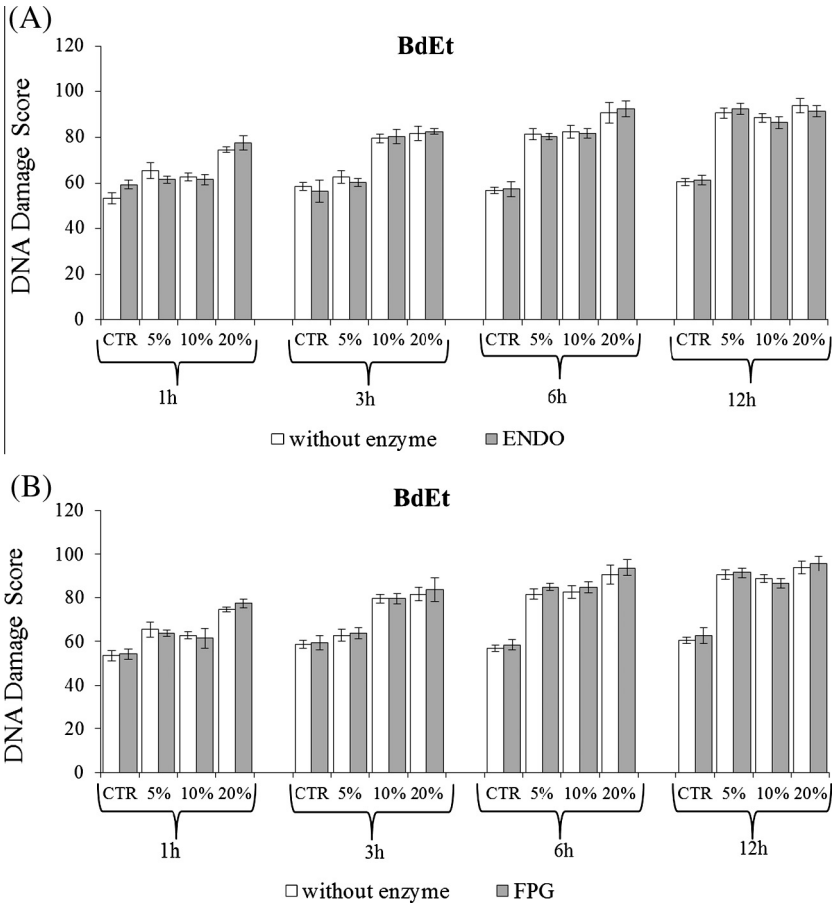

Fig. 9. Score of DNA damage in ZFL cells exposed to 5%, 10% and 20% of BdEt for 1, 3, 6 and 12 h, quantified by the standard comet assay (without enzyme) and the comet assay with the use of the enzyme endonuclease III (A) and FPG (B). Results are mean ± SE of at least four independent experiments.

in both soluble fractions. The superfamily of glutathione S-transferases catalyzes the conjugation of xenobiotics with glutathione, rendering them more water soluble and thus facilitating their excretion prior to Phase I biotransformation, which can lead to the generation of ROS (Van der Oost et al., 2003). Living cells have several mechanisms to restore their original redox state after a temporary exposure to increased levels of ROS. The main mechanism of redox homeostasis is based on redox-sensitive signaling cascades that lead to augmented expression of antioxidant enzymes. In many cells, high concentrations of ROS induce the expression of genes whose products exhibit antioxidant activity. Now, if the increase in ROS is relatively small, the antioxidant response may suffice to offset it and restore the original equilibrium (Droge, 2002). The results of this study are more consistent with the second case, in which the increase in ROS production was detected in cells exposed to both methylic and ethylic Bd, but this increase seems not to have been sufficient to activate the genes responsible for the production of antioxidant enzymes. Augmented catalase was only found in cells exposed to dilutions of 10% and 20% of methylic Bd at 6 h, indicating that one of the increased ROS is hydrogen peroxide. On the other hand, it is also known that the substrate of some of the antioxidant enzymes can also influence the activity of others and an excess of superoxide anion may be responsible for decreased CAT activity (Modesto and Martinez, 2010). In the case of the present study the reduction in CAT activity may be due to an excess of superoxide ions, which were probably not being neutralized efficiently by SOD, and might be responsible in part for the increased ROS production detected after 12 h of exposure to 10% and 20% BdMt.

The degree of DNA integrity has been proposed as a sensitive indicator of genotoxicity and an effective biomarker for environmental monitoring. DNA damage of aquatic organisms living in polluted environments can be used to study the genotoxicity of toxic agents, as well as in the assessment of ecotoxicological and environmental risks (Zhu et al., 2005). Several genetic mechanisms have been monitored in fish cells lines to evaluate the genotoxicity of substances in aquatic environments. The comet assay has received considerable attention and has been used successfully in several fish cell lines (Bols et al., 2005). However, the literature still contains no report of the comet assay performed with the ZFL cell line. Our results indicated that both the methylic and ethylic Bd were genotoxic to ZFL cells.

In the alkaline version of the standard comet assay, DNA lesions were found in the cells exposed to BdMt in all the experimental conditions, while ZFL cells exposed to BdEt showed no DNA damage in the cells exposed to the lowest dilution. DNA lesions detected by the comet assay are double and single-strand breaks and alkali labile sites (Tice et al., 2000). The sensitivity of the comet assay can be enhanced by adding restriction endonucleases to the process, which recognize lesions in DNA and convert these non-repaired lesions into additional DNA breaks (Kienzler et al., 2012). With the use of these endonucleases, it was possible to detect an increase in DNA damage when compared with the standard comet assay, in a 12-h period, in cells exposed to 20% BdMt. This increase is due to the appearance of breaks related with sites sensitive to the enzyme FPG. It is known that this enzyme detects oxidized purine bases such as 8-oxoguanine (Collins et al., 1996). It can be inferred that, at least in part, the damage found in the DNA

of cells exposed to BdMt is from oxidative origin. This hypothesis is supported by evidence of the increase in ROS production and the appearance of breaks related with sites sensitive to the enzyme FPG found in this study.

To avoid oxidative stress, cells are equipped with a set of antioxidant enzymes that keep the intracellular ROS at suitable levels (Zhang et al., 2008). However, the elevation of ROS found in the ZFL cells exposed to both types of biodiesel was not enough to activate the production of antioxidant enzymes. Therefore, to combat the DNA damage caused by ROS, it can be assumed that the repair system was activated, since the cells in the tested dilutions (5%, 10% and 20%) remained viable. Sandrini et al. (2009) reported the activation of two genes of ZFL repair system in cells exposed to ultraviolet light; one of them is Apex1, which is linked to the repair of oxidative lesions in DNA.

In conclusion, the response pattern of cells exposed to BdMt and BdEt was found to be similar, but the effects of BdMt appear to have been more intense. In general, both soluble fractions seem to be stressors for cells, leading them to activate a set of homeostatic control systems to compensate for perturbations and adapt to the stressor. The perturbations found in cells exposed to both soluble fractions were alterations in ROS production, activation of the biotransformation enzyme GST, and damage to the DNA molecule. BdMt was more cytotoxic than BdEt and promoted oxidative damage to the DNA molecule. Therefore, biodiesel produced by both the methylic and ethylic routes may cause cytotoxic, biochemical and genotoxic alterations in ZFL cells, but the type and intensity of the alterations found in ZFL cells appear to be dependent on the biodiesel production route.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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