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

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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

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system for studying 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 Q3 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