Toxicology in Vitro 30 (2015) 225–230

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/tiv

Effects of the water soluble fraction of gasoline on ZFL cell line: Cytotoxicity, genotoxicity and oxidative stress

Debora Lachner [a], Luciana F. Oliveira [a], Claudia B.R. Martinez [a,b,*]

[a] Programa de Pós-Graduação em Ciências Biológicas, Universidade Estadual de Londrina — UEL, Londrina, Paraná, Brazil
[b] Departamento de Ciências Fisiológicas, Universidade Estadual de Londrina — UEL, Londrina, Paraná, Brazil

ARTICLE INFO

Article history:
Received 17 June 2015
Received in revised form 2 November 2015
Accepted 11 November 2015
Available online 11 November 2015

Keywords:
Cell viability
Comet assay
Danio rerio
DNA damage
in vitro test
MTT assay

ABSTRACT

This work aimed to evaluate the effects of different dilutions of gasoline water-soluble fraction (GSF) on *Danio rerio* hepatocyte cell line (ZFL). Two tests were used to assess cell viability, MTT reduction assay (MTT) and the Trypan blue (TB) exclusion test. Oxidative stress was evaluated through the quantification of reactive oxygen species (ROS) and the assessment of the total antioxidant capacity against peroxyl radicals (ACAP) and the comet assay was employed to assess DNA damage. ZFL cells were exposed to 5, 10, 25 and 50% GSF or only to saline for 1, 3 and 6 h. The GSF exhibited concentration-dependent cytotoxicity, and longer exposure times resulted in lower cell viability as indicated by both MTT and TB assays. The establishment of oxidative stress in cells exposed to GSF was not observed at any exposure period and the lower ROS levels could be related to the increased antioxidant capacity after 6-hour exposure. DNA damage was significantly increased after exposure to GSF at the three experimental times. Taking together these results show that GSF has a genotoxic potential at the lower concentrations and becomes cytotoxic at higher concentrations and that ZFL can be considered a good biological model for in vitro toxicological studies.

1. Introduction

Water pollution caused by crude oil and its derivatives produces the highest impact in coastal and estuarine regions (Pacheco and Santos, 2001), and its occurrence is closely linked to human activity (Gao and Chen, 2008). More than 90,000 barrels of crude oil were consumed worldwide each day in 2013, according to the U.S. Energy Information Administration (EIA, 2015).

Gasoline is a crude oil derivative of great economic importance and its extensive use is troublesome because various events may promote contamination and consequent environmental damage caused by this fuel, including illegal dumping by oil refineries, transportation accidents, and inadequate storage (Xu et al., 2015). Besides, small gasoline spills frequently occur at gasoline dispensing stations (Hilpert and Breysse, 2014). Gasoline is essentially composed of hydrocarbons (Petrobras, 2014), and its water-soluble fraction is composed mainly of monocyclic and polycyclic aromatic chains (Fedato et al., 2010; Simonato et al., 2011). The toxic potential of gasoline for aquatic organisms is directly related to the most water-soluble aromatic hydrocarbons, i.e., benzene, toluene and xylene (BTEX hydrocarbons), and to some polyaromatic hydrocarbons such as naphthalene and phenanthrene (Tiburtius et al., 2004).

The permeability of hydrocarbons in tissues, including the skin and gastrointestinal tract (Adami et al., 2006; Lorenzi et al., 2012; Moro et al., 2013), and their ability to accumulate in sediments (David et al., 2010; Trisciani et al., 2011) stand out among the characteristics that facilitate the contact of aquatic organisms with these pollutants. Consequently, several adverse effects on aquatic biota have been noted, including apoptosis induction (Zhou et al., 2010), tissue necrosis (Akaishi et al., 2004), hormonal changes (Arukwe et al., 2008), plasma membrane destabilization (Camus et al., 2003), embryonic morphological changes (Debruyn et al., 2007), mutagenic and carcinogenic effects (Fedato et al., 2010; Simonato et al., 2011, 2008; Vanzella et al., 2007).

Satisfactory biomonitoring studies may be performed using in vitro biological models. The use of cell lines is one of the most promising methods because it shows fast, efficient, and representative responses and reduces the use of animals in research studies (Teng et al., 2013). Currently, immortalized liver cell lines are among the most widely used in vitro models for liver toxicity testing (Soldatow et al., 2013). The *Danio rerio* permanent hepatocyte cell line, ZFL, is a model of fish-derived cells considered relevant for the identification of toxicological hazard to aquatic organisms (Gajski et al., 2015). Several studies demonstrate the sensitivity of this cell line when exposed to various contaminants, including metals (Chan et al., 2006; Costa et al., 2012; Sandrini et al., 2009; Seok et al., 2007), pesticides (Goulart et al., 2015), pharmaceuticals (Bopp and Lettieri, 2008; Pomati et al., 2007; Gajski et al., 2015), effluents (Christianson-Heiska and Isomaa, 2008) and biodiesel (Cavalcante et al., 2014).

* Corresponding author at: Departamento de Ciências Fisiológicas, Universidade Estadual de Londrina, PB: 10011, Londrina, Paraná 86057-970, Brazil.
E-mail address: cbueno@uel.br (C.B.R. Martinez).

http://dx.doi.org/10.1016/j.tiv.2015.11.008
0887-2333/

However, the ZFL cell line, as many other immortalized cell lines, have altered gene expression and sensitivity to xenobiotics when compared to in vivo models (Eide et al., 2014); besides, cell lines of different fish species are not equivalent in terms of metabolic capacity (Creusot et al., 2014). ZFL cells seemed the less metabolically active ones in comparison to cell lines derived from topminnow (*Poeciliopsis lucida*) hepatoma (PLHC-1) and from rainbow trout liver (RTL-W1) (Creusot et al., 2014). On the other hand, ZFL cells were more susceptible to the genotoxic effects of cytostatic drugs than human-derived cells, showing that this cell line provides a relevant and sensitive tool to screen genotoxic potential of environmental pollutants in a hazard assessment approach (Gajski et al., 2015).

Considering the toxic potential of hydrocarbons to aquatic organisms and the important role of fish cell lines in toxicological research, this work aimed to evaluate the possible cytotoxic, genotoxic, and biochemical effects of different dilutions of the water-soluble fraction of gasoline on ZFL cells.

2. Materials and methods

2.1. ZFL maintenance

ZFL cells subcultured in culture medium supplemented with Leibovitz L-15 medium (Gibco®), RPMI 1640 medium (Gibco®), and fetal bovine serum (FBS) (Gibco®) were maintained in a dry oven at 28 °C in 25-cm^2 flasks.

2.2. Preparation of the gasoline water-soluble fraction

The gasoline water-soluble fraction (GSF) was prepared by simulating, at the laboratory scale, a gasoline spill under tropical conditions, as described in Simonato et al. (2011), with small modifications. Gasoline and distilled water were added to a beaker at a 1:4 ratio. This mixture was exposed to direct solar radiation for 6 h and then placed in a dark box for 18 h. The aqueous phase (GSF) was separated and stored in a dark container at 4 °C until use, without exceeding 5 days of storage. The GSF resulting from this step was normalized to 100% concentration.

The initial 100% GSF solution was diluted in distilled water to 5, 10, 25 and 50% for cell exposure. The salts composing phosphate buffered saline (PBS: 2.68 mM KCl, 1.47 mM KH_2PO_4, 136.89 mM NaCl, and 8.10 mM anhydrous Na_2HPO_3) were added to the different GSF dilutions, and the pH of each dilution was adjusted to 7.4. Then, the dilutions supplemented with salts were filtered through a 0.22-μm filter (TPP®) and stored in a dark bottle at 4 °C until use, without exceeding 24 h.

2.3. Cytotoxicity tests

Two tests were used to assess cell viability, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay (MTT) and the Trypan blue (TB) exclusion test. Thus, 24-well plates were seeded at a density of approximately 10^5 cells per mL. Two independent experiments were conducted for each test, with six replicates per treatment in total. Following the 48-h incubation period, the cells were exposed to PBS only (control group — CTR) or to the GSF dilutions for 1, 3 or 6 h in a dry oven at 28 °C.

To perform the TB exclusion test, the cells were detached from the wells following contaminant exposure, and 0.4% Trypan blue dye was added. Subsequently, 100 cells were counted in a Neubauer chamber. Cell viability is expressed as the percentage of viable cells. The treatments that showed cell viability values equal to or higher than 80% (Tice et al., 2000) were selected to quantify the reactive oxygen species (ROS) and total antioxidant capacity against peroxyl radicals (ACAP) and to perform the comet assay.

The MTT assay was applied according to the method of Mosmann (1983), with modifications. Following the exposure times, the solutions were removed, and MTT salt (0.33 $mg \cdot mL^{-1}$) diluted in culture medium was added to each well. The plates were then incubated for 4 hours in a dry oven at 28 °C. After completing this step, the culture medium was removed, and dimethyl sulfoxide (DMSO, 99.5%) was added to dilute the formazan. The final volume was transferred to a 96-well plate, and a spectrofluorometer (VICTOR 3, PerkinElmer) reading was performed at 540 nm. The samples exposed to PBS only (control group — CTR) were considered to show 100% cell viability (CV). The CV values of samples exposed to the GSF solutions were calculated proportional to the CTR sample.

2.4. Biochemical assays

Cell exposure was performed in cell culture flasks due to the need for higher protein concentrations for the quantification of reactive oxygen species (ROS), assessment of the total antioxidant capacity against peroxyl radicals (ACAP), and the comet assay. Therefore, the flasks were seeded at a density of approximately 10^6 cells per mL to obtain a homogeneous and confluent monolayer.

For exposure to the GSF solutions, the flasks were washed with PBS, and then, the different GSF dilutions were added, while the control flasks contained PBS only. After completing this step, the culture flasks were incubated at 28 °C for 1, 3, or 6 h. After exposure to the GSF, the test solution was discarded and the cells were rinsed with trypsin (0.125%), which subsequently was inactivated with PBS solution and 10% FBS. The cell suspension of each flask was transferred to microcentrifuge tubes and centrifuged (137 *g*, 4 °C, 7 min). The supernatant was then discarded, and the cells were resuspended in PBS.

The protocol established by Amado et al. (2009), with modifications, was used to perform the ROS and ACAP tests. First, the total protein concentration was determined (Bradford, 1976). Then, the samples were diluted to a concentration of 100 $\mu g \cdot mL^{-1}$ in the reaction medium (30 mM HEPES, 200 mM KCl, and 1 mM $MgCl_2$, pH 7.2) in a black 96-well plate. The samples were aliquoted in quadruplicate, and two wells were treated with the compound 2,2′-azobis(2-amidinopropane) dihydrochloride (10 mM ABAP, 0.1 M K^+, pH 7.2), which generates peroxyl radicals. The other two wells received potassium phosphate buffer (0.1 M K^+, pH 7.4) only. Then, an autofluorescence reading was performed. Subsequently, 2′,7′-dichlorofluorescin diacetate (1 mM H_2DCF-DA) was added, and readings were performed at 35 °C (ex: 485 nm, em: 520 nm) for 30 min in 5-min intervals (Amado et al., 2009). H_2DCF-DA is cleaved by the sample esterases and releases H_2DCF, which reacts with reactive oxygen species, releasing dichlorofluorescein, a fluorescent compound. Thus, a higher concentration of reactive oxygen species will produce a higher intensity of fluorescence (Amado et al., 2009).

To quantify ROS and ACAP, the fluorescence data were fit to a second-degree polynomial function, and the value of the integral was calculated (Amado et al., 2009). The integral values of samples treated only with phosphate buffer were analyzed to calculate the amount of ROS. The difference between the areas of samples treated and not treated with ABAP was calculated to assess the ACAP. A greater difference between areas indicated a smaller antioxidant capacity of the sample. The ACAP data were inverted (1/relative area) to facilitate visualization of the results.

2.5. Genotoxicity assay

The comet assay was performed according to Singh et al. (1988), with modifications. After exposure, the samples (20 μL) were added to low-melting-point agarose, and the mixture was homogenized and split between two slides previously prepared with agarose, which were then covered with coverslips and placed in a refrigerator. Approximately 30 min later, the coverslips were removed, and the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, and 1% Triton X-100, pH 10.0) for one hour. Following this step, the slides were placed in an electrophoresis chamber and remained

covered with electrophoresis solution (10 M NaOH and 200 mM EDTA, pH > 13) for 30 min. Subsequently, electrophoresis was performed for 20 min at 25 V and 300 mA. Following this step, the slides were neutralized (0.4 M Tris, pH 7.5) and fixed in 100% ethanol for 10 min.

The coded slides were stained with gel red (Biotium®) and analyzed using a fluorescence microscope (Leica®, DM 2500) at 400× magnification. A total of 100 nucleoids were analyzed per sample. DNA damage was categorized into four classes, according to the size of the tail formed by the DNA fragments (Kobayashi et al., 1995): class 0, no apparent damage; class 1, a tail smaller than the nucleoid diameter; class 2, a tail one to two times larger than the nucleoid diameter; and class 3, a tail larger than twice the nucleoid diameter. The frequency of classes and the total number of damaged nucleoids in each sample were also calculated.

2.6. Statistical analysis

For each parameter analyzed, the results obtained for the different GSF dilutions at the different experimental times (1, 3 and 6 h) were compared using two-factor analysis of variance (Factor 1, dilution; Factor 2, time). The Student–Newman–Keuls (SNK) multiple comparison test was applied if significant differences were found. The variation of the results obtained with different dilutions at the same experimental times was analyzed using Pearson's correlation coefficient (r), and only the results with significant r values are shown. P values < 0.05 were considered statistically significant.

3. Results

3.1. Cytotoxicity

Table 1 outlines the CV results following exposure to GSF dilutions obtained using the TB exclusion test. A significant reduction in the CV was observed at 1 h only in cells exposed to the highest GSF concentration (50%); at 3 h, the CV decreased in cells exposed to the 25% and 50% GSF dilutions, and at 6 h, the CV was significantly decreased in cells exposed to the 10%, 25% and 50% GSF dilutions. It was also noted that the CV was significantly decreased with increases in the time of exposure to both the 25% and 50% GSF dilutions. Based on these results, the 5%, 10%, and 25% dilutions of GSF were selected to perform the ROS, ACAP, and comet assays because they showed a mean CV higher than 80%.

The results regarding the CV assessed using the MTT assay following cell exposure to the GSF are outlined in Table 2. The CV values were significantly lower at all times tested after exposure to the 10%, 25% and 50% dilutions of the GSF and reached zero after exposure to the 50% GSF dilution for 6 h. Only ZFL cells exposed to the 5% GSF dilution for 3 and 6 h showed significantly increased CV values.

Overall, a significant decrease in the CV was observed during longer exposure times. Additionally, the CV values decreased as the concentration of GSF increased, in a concentration-dependent manner, as evidenced by the significant values of r, which were noted both in the TB and MTT assays (Tables 1 and 2).

Table 1
Cell viability (%) using the Trypan blue (TB) exclusion test for ZFL cells exposed to PBS (CTR) or to different dilutions of gasoline soluble fraction (GSF): 5, 10, 25 and 50%, for 1, 3 and 6 h.

Cell viability—TB test (%)			
	1 h	3 h	6 h
CTR	94.2 ± 1.7 aA	93.8 ± 1.3 aA	97.6 ± 1.6 aA
5%	93.8 ± 1.3 aA	94 ± 1.5 aA	96.2 ± 0.8 aA
10%	92.2 ± 1.9 aA	93.4 ± 1.5 aA	92.2 ± 1.9 bA
25%	90 ± 2.2 aA	87.4 ± 2.0 bB	82.4 ± 2.7 cB
50%	77.8 ± 3.9 bA	66.6 ± 2.5 cB	59.6 ± 6.4 dC
	r = −0.971	r = −0.964	r = −0.995

Results are mean ± SD; r indicates the Pearson's correlation coefficient between CV and treatment, for each exposure time. Different lowercase letters indicate significant difference between treatments at the same exposure time; different uppercase letters indicate significant difference between exposure times to the same treatment (P < 0.05).

Table 2
Cell viability (%) using the MTT assay for ZFL cells exposed to PBS (CTR) or to different dilutions of gasoline soluble fraction (GSF): 5, 10, 25 and 50%, for 1, 3 and 6 h.

Cell viability—MTT assay (%)			
	1 h	3 h	6 h
CTR	100.0 ± 0.0 aA	100.0 ± 0.0 aA	100.0 ± 0.0 aA
GSF 5%	88.5 ± 2.1 bA	111.6 ± 4.2 bB	126.3 ± 2.6 bC
GSF 10%	81.8 ± 3.2 cA	80.9 ± 4.0 cA	66.8 ± 5.6 cB
GSF 25%	34.4 ± 5.8 dA	18.2 ± 2.6 dB	17.0 ± 4.7 dB
GSF 50%	11.2 ± 0.9 eA	3.2 ± 0.8 eB	0.0 eC
	r = −0.971	r = −0.926	r = −0.892

Results are mean ± SD; r indicates the Pearson's correlation coefficient between CV and treatment, for each exposure time. Different lowercase letters indicate significant difference between treatments at the same exposure time; different uppercase letters indicate significant difference between exposure times to the same treatment (P < 0.05).

3.2. ROS and ACAP

After 1 h exposure, ROS generation decreased significantly with increasing concentrations of the GSF. On the other hand, after 3 h, a significant increase in the ROS levels was observed in the samples treated with the GSF, which peaked at 10%. Over again, at 6 h, the cells exposed to GSF showed significant lower concentrations of ROS than the cells exposed to PBS only (CTR) (Table 3).

After 1 h exposure to GSF, at all concentrations tested, ZFL cells showed ACAP values significantly lower than control cells. In contrast, ZFL cells exposed to 10% and 25% GSF for 3 h showed ACAP values significantly higher than control cells and ZFL cells exposed to 5% GSF. At 6 h exposure, ZFL cells exposed to all GSF concentrations showed significantly higher ACAP values than the control cells (Table 4). Furthermore, when comparing the ACAP values at different exposure times to the same treatment, a significant increase was observed at 6 h, except in the cells exposed to PBS only (CTR).

3.3. Genotoxicity

DNA damage was significantly increased after ZFL exposure to 5% and 10% GSF dilutions compared to the control condition at the three experimental times (Table 5). Despite showing CV values higher than 80%, the DNA damage upon exposure to the 25% GSF dilution was extensive, regardless of exposure time, and most nucleoids exhibited DNA scattered into cloud shapes. These nucleoids were not counted. Furthermore, the Pearson correlation coefficient (r), applied between the damage score and GSF dilutions, for each exposure time, showed significant values (1 h: 0.989; 3 h: 0.988; 6 h: 0.985), demonstrating the concentration-dependent relationship between these parameters, i.e., as the GSF concentration increased, the ZFL DNA damage intensified. Furthermore, for each treatment, a significant increase in the damage scores was observed with the increase of the exposure time (Table 5).

Table 3
Production of reactive oxygen species (ROS) in ZFL cells exposed to PBS (CTR) or to different dilutions of gasoline soluble fraction (GSF): 5, 10, 25 and 50%, for 1, 3 and 6 h.

ROS production Area (UF × min) × 10^4			
	1 h	3 h	6 h
CTR	5.43 ± 0.95 aA	2.83 ± 0.86 aB	7.19 ± 1.09 aC
GSF 5%	4.19 ± 0.79 bA	4.01 ± 1.01 bA	4.91 ± 0.62 bA
GSF 10%	2.23 ± 0.68 cA	7.97 ± 0.97 cB	4.67 ± 0.97 bC
GSF 25%	2.29 ± 0.47 cA	4.71 ± 1.10 bB	4.18 ± 0.52 bB

Results are mean ± SD. Different lowercase letters indicate significant difference between treatments at the same exposure time; different uppercase letters indicate significant difference between exposure times to the same treatment (P < 0.05).

Table 4
Antioxidant capacity against peroxyl radicals (ACAP) of ZFL cells exposed to PBS (CTR) or to different dilutions of gasoline soluble fraction (GSF): 5, 10, 25 and 50%, for 1, 3 and 6 h.

ACAP (1/relative area) × 10^2			
	1 h	3 h	6 h
CTR	5.43 ± 0.87 aA	2.11 ± 0.28 aB	6.32 ± 0.16 aA
GSF 5%	4.14 ± 0.78 bA	2.25 ± 0.13 aB	12.12 ± 0.18 bC
GSF 10%	3.59 ± 0.21 bA	3.30 ± 0.48 bA	11.02 ± 0.26 bB
GSF 25%	4.53 ± 0.95 bA	3.67 ± 0.96 bA	12.46 ± 1.67 bB

Results are mean ± SD. Different lowercase letters indicate significant difference between treatments at the same exposure time; different uppercase letters indicate significant difference between exposure times to the same treatment ($P < 0.05$).

In addition, regarding exposure to the GSF, Table 6 shows the percentage of nucleoids observed in each comet class and the total number of damaged nucleoids. Overall, the data indicate an increase in ZFL cells with damaged nucleoids after exposure to 5% and 10% GSF dilutions and a higher incidence of comet class 2 and 3 in cells exposed to the GSF, with a significant increase in the frequency of damaged nucleoids with exposure time in all treatments.

The analysis of the interaction between the two factors analyzed, time and concentration, was statistically significant in all treatments.

4. Discussion

In the assays used, a significant variation in the parameters tested at the different exposure times was observed in the control cells. These differences most likely resulted from exposure to PBS because this solution does not have the nutrients required by ZFL and, therefore, may be a source of stress to cells, especially at longer exposure times.

The GSF exhibited concentration-dependent cytotoxicity, and longer exposures resulted in lower cell viability in cells exposed to this contaminant, which was observed in both tests used in this study. Thus, at low concentrations, including 5% and 10% GSF, the pollutant showed no cytotoxic effect, which changed with increasing concentrations. Similar results were found by Fent and Bätscher (2000) after exposing the fish hepatoma cell line PLHC-1 to several polycyclic aromatic hydrocarbons (PAH). The same pattern was observed by Kammann et al. (2001) and Yang et al. (2010) when exposing different fish cell lines, including gill (FG) and skin cells (*Epithelioma papulosum cyprini*, EPC), to marine sediment extracts contaminated by PAH. The mortality of cells at high concentrations of hydrocarbons may be related to the induction of apoptosis through the formation of DNA adducts (Zhou et al., 2010). The chemical characteristics of the GSF produced by the same type of spill simulation were already reported by Simonato et al. (2011). These authors showed that GSF consists of high levels of monoaromatic hydrocarbons, mainly of ethylbenzene, xylene and benzene, and among the PAH analyzed; naphthalene was the most significant, followed by anthracene and phenanthrene.

Table 5
DNA damage score in ZFL cells exposed to PBS (CTR) or to different dilutions of gasoline soluble fraction (GSF): 5, 10, 25 and 50%, for 1, 3 and 6 h.

DNA damage score			
	1 h	3 h	6 h
CTR	89.5 ± 18.6 aA	54.6 ± 16.0 aB	125.6 ± 13.7 aC
GSF 5%	179.5 ± 39.9 bA	115.6 ± 6.2 bB	224.0 ± 19.7 bC
GSF 10%	233 ± 33.4 cA	222.0 ± 4.6 cA	276.4 ± 14.1 cB
	r = 0989	r = 0.988	r = 0.985

Results are mean ± SD. r indicates the Pearson's correlation coefficient between the damages scores and the treatment, for each exposure time Different lowercase letters indicate significant difference between treatments at the same exposure time; different uppercase letters indicate significant difference between exposure times to the same treatment ($P < 0.05$).

Table 6
Frequency (%) of nucleoids observed in each comet class (0, 1, 2, 3) and the total number of damaged nucleoids (mean ± SD, n = 5) in ZFL cells exposed to PBS (CTR) or to different dilutions of gasoline soluble fraction (GSF): 5 and 10%, for 1, 3 and 6 h.

Exposure time	Group	Class				Damaged nucleoids
		0	1	2	3	
	CTR	34.67	43.67	19.17	2.50	62.8 ± 13.6 aA
1 h	5%	6.00	32.83	36.83	24.33	95.6 ± 4.1 bA
	10%	0.33	12.33	41.33	46.00	99.6 ± 0.5 bA
	CTR	64.00	21.00	11.40	3.60	36.0 ± 10.3 aB
3 h	5%	19.80	46.60	31.80	1.80	83.6 ± 7.7 bB
	10%	0.00	11.00	56.00	33.00	100.0 ± 0.0 cA
	CTR	19.20	47.00	22.80	11.00	80.8 ± 3.0 aC
6 h	5%	3.80	18.20	28.20	49.80	96.2 ± 5.2 bC
	10%	0.20	2.20	18.60	79.00	99.8 ± 0.4 bB

Different lowercase letters indicate significant difference between treatments at the same exposure time; different uppercase letters indicate significant difference between exposure times to the same treatment ($P < 0.05$).

Specifically, regarding the MTT assay, an increase in the metabolic rate was observed when the cells were exposed to 5% GSF, which may suggest hormesis. This phenomenon is characterized by the stimulation of the parameter assessed at low stressor concentrations and its inhibition at high concentrations, resulting in biphasic response curves, and is often observed regardless of the chemical or physical agents, biological models, or biomarkers evaluated (Bain and Kumar, 2014; Calabrese and Baldwin, 2002; Llabjani et al., 2014). This phenomenon may be related to the activation of the cellular homeostasis adaptive pathway and to cell, organ, or system protection against unfavorable environments, which requires an energy shift, leading to changes in several basal functions (Zhang et al., 2008).

The establishment of oxidative stress in cells exposed to GSF was not observed at any exposure period. Furthermore, the lower ROS levels could be related to the increased antioxidant capacity (AOC), which was significantly increased at 6 h. The activation of antioxidant defense mechanisms was also observed in ZFL exposed to copper for 96 h (Chen and Chan, 2011). Furthermore, the efficiency of antioxidants in preventing cell damage was demonstrated, both in ZFL cells (Seok et al., 2007) and in the human hepatoma cell line HepG2 (Zhou et al., 2010). The cellular antioxidant mechanisms against ROS were clearly activated at 6 h of exposure to GSF, regardless of dilution. This finding suggests that the antioxidant mechanisms of ZFL cells require some time to become fully activated and responsive.

The high genotoxic potential of the GSF was observed because the damage scores of the different treatments reached values near the maximum (300). A concentration-dependent relationship was also observed between damage and dilution. The frequency data of damaged nucleoids corroborate this hypothesis because the most common classes were 2 and 3, in which DNA repair is considered difficult. This response pattern was also observed by Kammann et al. (2001) and Yang et al. (2010) when assessing the toxic effects of sediments contaminated with PAH in different cell lines. Furthermore, both the increase in DNA damage and the formation of micronucleated cells were observed in human lung carcinoma cells (A549) following exposure to particulate matter extract contaminated with gasoline (Zhang et al., 2008), suggesting the difficulty of DNA damage repair caused by this pollutant. When the in vivo experiments were compared, the same findings were observed by Caliani et al. (2009), who exposed the fish *Gambusia affinis* to water containing mainly PAH and found increased number of micronucleated cells after 30 days of exposure. Consistent with these findings, Moro et al. (2013) observed increased DNA damage and micronucleus rates as well as increased oxidative protein damage in individuals working in gas stations with exposure to hydrocarbons daily. However, in the present work, the data regarding the levels of ROS and the ACAP suggest that the DNA damage on ZFL cells caused by exposure to the GSF most likely had no oxidative origin.

The results obtained in this study, using ZFL cell line, clearly show that GSF has a genotoxic potential at the lower concentrations and becomes cytotoxic at higher concentrations. Some other considerations also apply, including the claim that the in vitro test model used meets the requirements to be considered a good biological model for toxicological studies, including sensitivity and responsiveness as well as reduced cost and waste production.

Conflict of interest statement

None.

Acknowledgments

This work is part of the Master Dissertation of D. Lachner. CNPq (Grant number: 573949/2008-5) and Fundação Araucária (Grant number: 419/2009-14755) provided financial support for this study. C.B.R. Martinez is a research fellow from CNPq and member of the National Institute of Science and Technology in Aquatic Toxicology (INCT-TA, CNPq 573949/2008-5).

References

Adami, G., Larese, F., Venier, M., Barbieri, P., Lo Coco, F., Reisenhofer, E., 2006. Penetration of benzene, toluene and xylenes contained in gasolines through human abdominal skin in vitro. Toxicol. in Vitro 20, 1321–1330.

Akaishi, F.M., Silva-De-Assis, H.C., Jakobi, S.C.G., Eiras-Stofella, D.R., St-Jean, S.D., Courtenay, S.C., 2004. Morphological and neurotoxicological findings in tropical freshwater fish (*Astyanax* sp.) after waterborne and acute exposure to water soluble fraction (WSF) of crude oil. Arch. Environ. Contam. Toxicol. 46, 244–253.

Amado, L.L., Garcia, M.L., Ramos, P.B., Freitas, R.F., Zafalon, B., Ferreira, J.L.R., Yunes, J.S., Monsserat, J.M., 2009. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: application to evaluate microcystins toxicity. Sci. Total Environ. 407, 2115–2123.

Arukwe, A., Nordtug, T., Kortner, T.M., Mortensen, A.S., Brakstad, O.G., 2008. Modulation of steroidogenesis and xenobiotic biotransformation responses in zebrafish (*Danio rerio*) exposed to water-soluble fraction of crude oil. Environ. Res. 107, 362–370.

Bain, P.A., Kumar, A., 2014. Cytotoxicity of binary mixtures of human pharmaceuticals in a fish cell line: approaches for non-monotonic concentration–response relationships. Chemosphere 108, 334–342.

Bopp, S., Lettieri, T., 2008. Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line. BMC Pharmacol. 8, 8.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Calabrese, E.J., Baldwin, L.A., 2002. Hormesis: the dose–response revolution. Annu. Rev. Pharmacol. Toxicol. 43, 175–197.

Caliani, I., Porcelloni, S., Mori, G., Frenzilli, G., Ferraro, M., Marsili, L., Casini, S., Fossi, M.C., 2009. Genotoxic effects of produced water in mosquito fish (*Gambusia affinis*). Ecotoxicology 18, 75–80.

Camus, L., Birkely, S.R., Jones, M.B., Børseth, J.F., Grøsvik, B.E., Gulliksen, B., Lønne, O.J., Regoli, F., Depledge, M.H., 2003. Biomarker responses and PAH uptake in *Mya truncata* following exposure to oil-contaminated sediment in an Arctic fjord (Svalbard). Sci. Total Environ. 308, 221–234.

Cavalcante, D.G.S.M., da Silva, N.D.G., Marcarini, J.C., Mantovani, M.S., Marin-Morales, M.A., Martinez, C.B.R., 2014. Cytotoxic, biochemical and genotoxic effects of biodiesel produced by different routes on ZFL cell line. Toxicol. in Vitro 28, 1117–1125.

Chan, K.M., Ku, L.L., Chan, P.C.Y., Cheuk, W.K., 2006. Metallothionein gene expression in zebrafish embryo-larvae and ZFL cell-line exposed to heavy metal ions. Mar. Environ. Res. 62, S83–S87.

Chen, D.S., Chan, K.M., 2011. Differentially expressed proteins in zebrafish liver cells exposed to copper. Aquat. Toxicol. 104, 270–277.

Christianson-Heiska, I., Isomaa, B., 2008. The use of primary hepatocytes from brown trout (*Salmo trutta lacustris*) and the fish cell lines RTH-149 and ZF-L for in vitro screening of (anti)estrogenic activity of wood extractives. Toxicol. in Vitro 22, 589–597.

Costa, C.L.A., Chaves, I.S., Ventura-Lima, J., Ferreira, J.L.R., Ferraz, L., Carvalho, L.M., Monserrat, J.M., 2012. *In vitro* evaluation of co-exposure of arsenium and an organic nanomaterial (fullerene, C_{60}) in zebrafish hepatocytes. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 155, 206–212.

Creusot, N., Brion, F., Piccini, B., Budzinski, H., Porcher, J.M., Aït-Aïssa, S., 2014. BFCOD activity in fish cell lines and zebrafish embryos and its modulation by chemical ligands of human aryl hydrocarbon and nuclear receptors. Environ. Sci. Pollut. Res. http://dx.doi.org/10.1007/s11356-014-3882-8.

David, A., Gomez, E., Aït-Aïssa, S., Rosain, D., Casellas, C., Fenet, H., 2010. Impact of urban wastewater discharges on the sediments of a small Mediterranean river and associated coastal environment: assessment of estrogenic and dioxin-like activities. Arch. Environ. Contam. Toxicol. 58, 562–575.

Debruyn, A.M.H., Wernick, B.G., Stefura, C., Mcdonald, B.G., Rudolph, B.L., Patterson, L., Chapman, P.M., 2007. *In situ* experimental assessment of Lake Whitefish development following a freshwater oil spill. Environ. Sci. Technol. 41, 6983–6989.

EIA, Energy Information Administration (EIA, USA), 2015. Available at: <http://www.eia.gov/cfapps/ipdbproject/iedindex3.cfm?tid=5&pid=5&aid=2&cid=ww,&syid=2009&eyid=2013&unit=TBPD>.

Eide, M., Rusten, M., Male, R., Jensen, K.H.M., Goksøyr, A., 2014. A characterization of the ZFL cell line and primary hepatocytes asin vitro liver cell models for the zebrafish (*Danio rerio*). Aquat. Toxicol. 147, 7–17.

Fedato, R.P., Simonato, J.D., Martinez, C.B.R., Sofia, S.H., 2010. Genetic damage in the bivalve mollusk *Corbicula fluminea* induced by the water-soluble fraction of gasoline. Mutat. Res. 700, 80–85.

Fent, K., Bätscher, R., 2000. Cytochrome P4501A induction potencies of polycyclic aromatic hydrocarbons in a fish hepatoma cell line: demonstration of additive interactions. Environ. Toxicol. Chem. 19, 2047–2058.

Gajski, G., Gerić, M., Žegura, B., Novak, M., Nunić, J., Bajrektarević, D., Garaj-Vrhovac, V., Filipič, M., 2015. Genotoxic potential of selected cytostatic drugs in human and zebrafish cells. Environ. Sci. Pollut. Res. http://dx.doi.org/10.1007/s11356-015-4592-6.

Gao, X., Chen, S., 2008. Petroleum pollution in surface sediments of Daya Bay, South China, revealed by chemical fingerprinting of aliphatic and alicyclic hydrocarbons. Estuar. Coast. Shelf Sci. 80, 95–102.

Goulart, T.L.S., Boyle, R.T., Souza, M.M., 2015. Cytotoxicity of the association of pesticides roundup Transorb® and Furadan 350 SC® on the zebrafish cell line, ZF-L. Toxicol. in Vitro 29, 1377–1384.

Hilpert, M., Breysse, P.N., 2014. Infiltration and evaporation of small hydrocarbon spills at gas stations. J. Contam. Hydrol. 170, 39–52.

Kammann, U., Bunke, M., Steinhart, H., Theobald, N., 2001. A permanent fish cell line (EPC) for genotoxicity testing of marine sediments with the comet assay. Mutat. Res. 498, 67–77.

Kobayashi, H., Sugiyama, C., Morikawa, Y., Hayashi, M., Sofuni, T., 1995. A comparison between manual microscopic analysis and computerized image analysis in the single cell gel electrophoresis. M. M. Commun. 3, 103–115.

Llabjani, V., Hoti, V., Pouran, H.M., Martin, F.L., Zhang, H., 2014. Bimodal responses of cells to trace elements: insights into their mechanism of action using a biospectroscopy approach. Chemosphere 112, 377–384.

Lorenzi, D., Entwistle, J., Cave, M., Wragg, J., Dean, J.R., 2012. The application of an in vitro gastrointestinal extraction to assess the oral bioaccessibility of polycyclic aromatic hydrocarbons in soils from a former industrial site. Anal. Chim. Acta 735, 54–61.

Moro, A.M., Charão, M.F., Brucker, N., Durgante, J., Baierle, M., Bubols, G., Goethel, G., Fracasso, R., Nascimento, S., Bulcão, R., Gauer, B., Barth, A., Bochi, G., Moresco, R., Gioda, A., Salvador, M., Farsky, S., Garcia, S.C., 2013. Genotoxicity and oxidative stress in gasoline station attendants. Mutat. Res. 754, 63–70.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Pacheco, M., Santos, M.A., 2001. Biotransformation, endocrine, and genetic responses of *Anguilla anguilla* L. to petroleum distillate products and environmentally contaminated waters. Ecotoxicol. Environ. Saf. 49, 64–75.

Petrobras, 2014. Available at: < http://www.br.com.br/wps/portal/portalconteudo/home/!ut/p/c5/04_SB8K8xLLM9MSSzPy8xBz9CP0os3hLf0N_P293QwN3d09XAyNTb5_AwKBgQwMDA30_j_zcVP2CbEdFAHXS8qI!/dl3/d3/L2dBISEvZ0FBIS9nQSEh/?WCM_GLOBAL_CONTEXT=>.

Pomati, F., Cotsapas, C.J., Castiglioni, S., Zuccato, E., Calamari, D., 2007. Gene expression. Profiles in zebrafish (*Danio rerio*) liver cells exposed to a mixture of pharmaceuticals at environmentally relevant concentrations. Chemosphere 70, 65–73.

Sandrini, J.Z., Bianchini, A., Trindade, G.S., Nery, L.E.M., Marins, L.F.F., 2009. Reactive oxygen species generation and expression of DNA repair-related genes after copper exposure in zebrafish (*Danio rerio*) ZFL cells. Aquat. Toxicol. 95, 285–291.

Seok, S.H., Baek, M.W., Lee, H.Y., Kim, D.J., Na, Y.R., Noh, K.J., Park, S.H., Lee, H.K., Lee, B.H., Ryu, D.Y., Park, J.H., 2007. Arsenite-induced apoptosis is prevented by antioxidants in zebrafish liver cell line. Toxicol. in Vitro 21, 870–877.

Simonato, J.D., Guedes, C.L.B., Martinez, C.B.R., 2008. Biochemical, physiological and histological changes in the neotropical fish *Prochilodus lineatus* exposed to diesel oil. Ecotoxicol. Environ. Saf. 69, 112–120.

Simonato, J.D., Martinez, C.B.R., Fernandes, M.N., 2011. Gasoline effects on biotransformations and atioxidant defenses of the freshwater fish *Prochilodus lineatus*. Ecotoxicology 20, 1400–1410.

Singh, N.P., Mccoy, M.T., Tice, R.R., Schneider, E.L., 1988. A single technique for quantification of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.

Soldatow, V.Y., LeCluyse, E.L., Griffith, L.G., Rusyn, I., 2013. In vitro models for liver toxicity testing. Toxicol. Res. 2, 23–39.

Teng, Q., Ekman, D.R., Huang, W., Collete, T.W., 2013. Impacts of 17α-ethynylestradiol exposure on metabolite profiles of zebrafish (*Danio rerio*) liver cells. Aquat. Toxicol. 130, 184–191.

Tiburtius, E.R.L., Peralta-Zamora, P., Leal, E.S., 2004. Contaminação de águas por BTEX e processos utilizados na remediação de sítios contaminados. Quim. Nova 27, 441–446.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–221.

Trisciani, A., Corsi, I., Torre, C.D., Perra, G., Focardi, S., 2011. Hepatic biotransformation genes and enzymes and PAH metabolites in bile of common sole (*Solea solea*, Linnaeus, 1758) from an oil-contaminated site in the Mediterranean Sea: a field study. Mar. Pollut. Bull. 62, 806–814.

Vanzella, T.P., Martinez, C.B.R., Cólus, I.M.S., 2007. Genotoxic and mutagenic effects of diesel oil water soluble fraction on a neotropical fish species. Mutat. Res. 631, 36–43.

Xu, Z., Chai, J., Wu, Y., Qin, R., 2015. Transport and biodegradation modeling of gasoline spills in soil–aquifer system. Environ. Earth Sci. 74, 2871–2882.

Yang, F., Zhang, Q., Guo, H., Zhang, S., 2010. Evaluation of cytotoxicity, genotoxicity and teratogenicity of marine sediments from Qingdao coastal areas using in vitro fish cell assay, comet assay and zebrafish embryo test. Toxicol. in Vitro 24, 2003–2011.

Zhang, Q., Pi, J., Woods, C.G., Jarabek, A.M., Clewell, H.J., Andersen, M.E., 2008. Hormesis and adaptive cellular control systems. Dose–Response 6, 196–208.

Zhou, G.D., Richardson, M., Fazili, I.S., Wang, J., Donnelly, K.C., Wang, F., Moorthy, B., 2010. Role of retinoic acid in the modulation of benzo(a)pyrene-DNA adducts in human hepatoma cells: implications for cancer prevention. Toxicol. Appl. Pharmacol. 249, 224–230.