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Biodiesel from Soybean Promotes Cell Proliferation *in Vitro*

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Abstract

Toxicological responses of exhaust emissions of biodiesel are different due to variation in methods of generation and the tested biological models. A chemical profile was generated using ICP-MS and GC-MS for the biodiesel samples obtained in Brazil. A cytotoxicity assay and cytokine secretion experiments were evaluated in human bronchial epithelial cells (BEAS-2B). Cells were exposed to polar (acetone) and nonpolar (hexane) extracts from particles obtained from fuel exhaust: fossil diesel (B5), pure soybean biodiesel (B100), soybean biodiesel with additive (B100A) and ethanol additive (EtOH). Biodiesel and its additives exhibited higher organic and inorganic constituents on particles when compared to B5. The biodiesel extracts did not exert any toxic effect at concentrations 10, 25, 50, 75, and 100 $\mu\text{g mL}^{-1}$. In fact quite the opposite, a cell proliferation effect induced by the B100 and B100A extracts is reported. A small increase in concentrations of inflammatory mediators (Interleukin-6, IL-6; and Interleukin-8, IL-8) in the medium of biodiesel-treated cells was observed, however, no statistical difference was found. An interesting finding indicates that the presence of metals in the nonpolar (hexane) fraction of biodiesel fuel (B100) represses cytokine release in lung cells. This was revealed by the use of the metal chelator. Results suggest that metals associated with biodiesel's organic constituents might play a significant role in molecular mechanisms associated to cellular proliferation and immune responses.

Keywords

biodiesel; BEAS-2B; cytotoxicity; cytokines; cell proliferation; metal; particles; PAH

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1. Introduction

The energy consumed in the world is originated mostly from non-renewable sources such as natural gas, coal and oil; the latter two are very polluting and are large contributors to global warming. In this sense, there is a growing search for new renewable fuels. Therefore, biofuels are an interesting alternative by being a cleaner source of fuel energy.

In Brazil, ethanol and biodiesel are the two main biofuels used, which are produced mostly from sugar cane and soybean, respectively, although many other sources are also available, but used in a smaller scale. Biofuels have been used in the pure form or blended to commercial diesel (up to 7 % of biodiesel) and gasoline (up to 27 % of ethanol).

Although biofuels are biodegradable and a renewable source of energy, its possible health effect is under investigation, because gases and particles are emitted during its combustion. The chemical composition and level of pollutants released into the atmosphere changes according to the emission control technologies and fuel types employed, as a result, it is difficult to predict with certainty the effects without an extensive study of all emission sources. Information regarding biofuel exhaust particles, their chemical composition, size, morphology, biological and toxicological effects is scarce (Traviss et al., 2014). Biodiesel could present deleterious effects on the aquatic environments (da Cruz et al., 2012) and could exert undesirable effects on the human respiratory tract (Bünger et al., 2012). A review of literature showed that there are few toxicological, cytotoxicity and mutagenicity studies on the effects of biodiesel exhaust in biological systems. A very good extensive review on the emission and byproducts of biodiesel was published by Madden et al. (2011). Other studies using human lung cells (BEAS-2B) for toxicological assays after biodiesel exposure were reported (Steiner et al. 2013, Traviss et al., 2014; Gerlofs-Nijland et al., 2013; Hawley et al., 2014; Yanamala et al., 2013).

Here we evaluate and report the toxicological and immunological responses on human lung cells (BEAS-2B) after the exposure to biodiesel commonly used in Brazil. This research elucidates possible toxicological effects induced by particles from fuel and biofuel exhaust.

2. Materials and Methods

2.1 Sampling

The sampling system consisted of an acrylic chamber (1m × 1m × 1m) where the emissions were concentrated to avoid external contamination. This chamber was coupled to vacuum pumps with rotameters and connected to the filters, which collected total particulate matter (TPM). The emission generated by fuel burning was collected during the operation where a stationary diesel engine of cycle mono cylinder (pick-up vehicle, power 10 HP), was attached to a generator Toyama T6000 CXE3 1800 RPM/ 60 Hz set.

Four types of fuels were used in this study: 1) commercial diesel (fossil diesel with 5% of soybean biodiesel, B5), 2) pure soybean biodiesel (B100), 3) soybean biodiesel with butylhydroxyanisole (BHT) additive (B100A) and 4) ethanol additive: 91.06 % ethanol, 7.92 % alcohol nitrate tetrahydro-furfuryl (NTHF) and 0.99 % castor oil (EtOH). The

samplings were carried out during 30 minutes. Polycarbonate filters with porosity of 0.4 μm in diameter which is equal to 37 mm (Millipore HTPP 03700) were coupled to the vacuum pump at a flow rate of 10.0 mL min^{-1} . To determine the mass of TPM, filters were weighed before and after sampling on a precise balance (Mettler Toledo - Model XP 205, maximum capacity 220 g and readability 0.00001 g) at constant weight, until they presented standard deviation equal to or less than 0.00002 g.

2.2 Extraction Procedures

For toxicological assays, the filters containing TPM were sequentially extracted using hexane (HPLC grade) followed by acetone. The filters were cut into small pieces and extracted in 6 mL of each solvent under sonication for one hour. The resulting volume was evaporated under a gentle stream of nitrogen and the residue weighted. The residues of both solvents were diluted in appropriate media (described in section 2.4) in order to determine their cytotoxicity and cytokine secretion in BEAS-2B cells. Blank filters were prepared in the same way and in parallel with the samples.

In order to determine metal concentrations, the filters were treated with 3.0 mL of double distilled nitric acid digested for 2 hours at $95 \pm 5^\circ \text{C}$ (Mateus et al., 2013). Then the solutions were centrifuged to separate insoluble and diluted material. A reference material (NIST 1648a, urban dust), blank filters were also extracted following the same procedure. The extracts were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). The polycyclic aromatic hydrocarbon (PAH) associated with TPM was extracted with dichloromethane in an ultrasonic bath. Initially, filters were punched, placed in 50 mL flask, mixed with 20 mL of dichloromethane and stirred in ultrasonic for 20 min. The extracts were filtered to remove suspended particles. This procedure was repeated three times. The sum of the extracts was evaporated until reaching a final volume of 1.0 mL and then analyzed by gas chromatography.

2.3 Chemical Analysis

In this study an ICP-MS model NexIon 300X (Perkin Elmer/Sciex, USA) was used to determine elemental concentrations. The elements analyzed were Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, Ga, K, Mg, Mn, Na, Ni, Pb, Sb, Se, Ti, V and Zn. For the quantitative determination, calibration solutions prepared from multielement standard solutions of 1000 mg L^{-1} (Perkin Elmer 29 and Merck Titrisol) in nitric acid were used. The analytical standard curves were prepared with solution concentrations ranging from 5 $\mu\text{g L}^{-1}$ to 80 $\mu\text{g L}^{-1}$. Rhodium at concentration of 10 $\mu\text{g L}^{-1}$ was used as an internal calibration standard. The limit of detection (LOD) and quantification (LOQ) were determined considering $3 \times (\text{SD}/S)$ and $10 \times (\text{SD}/S)$, respectively, where SD is the standard deviation of the blanks and S the slope of analytical curve. This method was previously published and specific details on chemical analysis can be found in Mateus et al. 2013.

Analyses of PAH were performed in an ion trap GC/MS system (Finnigan Trace gas chromatographer coupled to a Finnigan Polaris mass spectrometer) with a column DB5MS (30 m $\times$ 0.25 mm $\times$ 0.25 μm). Sixteen PAH were quantified: naphthalene (NAF), acenaphthylene (Acen), acenaphthene (Ace), fluorene (Flu), phenanthrene (Phe), anthracene

(Ant), fluoranthene (Fluor), chrysene (Chr), pyrene (Pyr), benzo(a)pyrene (BaPy), benzo(e)pyrene (BePy), benz(a)anthracene (BaAnt), benzo(b)fluoranthene (BbFluor), benzo(k)fluoranthene (BkFluor), indeno(1,2,3-cd)pyrene (IP), dibenz(a,h)anthracene (DBzAhAnt), benzo(ghi)perylene (BghiPer), perylene (Per), dibenzothiophene (DBT); and methylated: alkylated naphthalenes (C1, C2, C3, and C4), fluorenes (C1, C2, and C3), phenanthrenes (C1, C2, C3, and C4), dibenzothiophenes (C1, C2, and C3), chrysenes (C1 and C2), and pyrenes (C1 and C2). The determination of PAH by GC/MS followed the EPA-8270D method and was also described previously (Massone et al., 2015).

2.4 Sample Preparation

The extract mass from fuel and biofuels was determined gravimetrically and subsequently dissolved in dimethylsulfoxide (DMSO). These fractions were constituted to a final stock solution of 100 mg mL⁻¹, which were used in the cell tests. Blank filters were analyzed in the same manner.

2.5 Cell Culture and Exposures

The BEAS-2B cell line was selected as a model because particles tend to deposit in the bronchial region in the respiratory airways such as bronchi (Becker et al., 2002; Mueller-Anneling et al., 2004). This cell type is suitable as being mostly affected by the interaction, deposition and toxic effects of air particle pollution. They have also been used to describe an important role in surveillance being easily activated by superficial exposure to microbial factors and are the most promising model to assess respiratory sensitization of chemical compounds (Diociaiuti et al., 2001; Vandebriel et al., 2011). Human bronchial epithelial cells (BEAS-2B) were obtained from the American Type Culture Collection (ATCC®CRL-9609™). Cells were cultured according to ATCC protocols with a slight modification using Keratinocyte growth medium (KGM-2, Lonza, Walkersville, MD, USA), maintained at 37 °C in humidified atmosphere of 5 % CO₂ and used at passages 44-59 as previously reported (Rodríguez-Cotto et al., 2014). The cells were seeded at a density of 5 × 10⁴ cells per well onto 96-well plates and incubated for 24 h. The extracts were diluted in cell media at concentrations ranging from 10 to 100 µg mL⁻¹ and used to expose cells for 24 h. An additional set of cells (n = 3) was concurrently exposed to extracts pre-treated with deferoxamine mesylate (DF, Sigma, Cat No D9533), a metal chelator, at a final concentration of 50 µmol L⁻¹. Deferoxamine mesylate is a trihydroxamic acid, siderophore secreted by *Streptomyces pilosus*, a fungus. It has been reported to chelate metals such as Pb, Cd, Ca, Mg in a moderate manner (Farkas et al., 2008) however, it binds with greater affinity to trivalent iron Fe (Swaran and Pachauri, 2010) and Al (Percy et al., 2011). It is used for Fe and Al clinical detoxification. All treatments were conducted at a final concentration of 0.01 % DMSO as a carrier or vehicle. Cell supernatants were collected after each exposure and used for cytokine analyses while the adhered cells to the plates were processed to evaluate cell viability.

2.6 Cytotoxicity

The Neutral Red bioassay was used to measure cell viability as previously described (Rodríguez-Cotto et al., 2013). Briefly, after a 24 h cell exposure to biodiesel extracts, the treatments were removed and BEAS-2B cells were incubated with Neutral Red dye (Sigma,

Cat No N2889) at a final concentration of $100 \mu\text{g mL}^{-1}$ for 3 h. The dye was then removed; cells were fixed in 1 % calcium chloride, 0.5 % formaldehyde, rinsed with phosphate buffered saline (PBS) and lysed using 1 % acetic acid and 50 % ethanol. Cell viability was spectrophotometrically determined at 540 nm using an Ultramark microplate reader (Bio Rad, Richmond, CA, USA). Triton-X ($25 \mu\text{g mL}^{-1}$) was used as a positive control for cell toxicity. The following controls were simultaneously employed in each experiment: media, media with deferoxamine and water. Cell viability less than 80 % was the cut off considered for cytotoxicity.

2.7 Cytokine Measurements

Cytokine analyses (IL-6, IL-8 and IL-10) were performed using a Fluorokine Multi-analyte Profiling Kit from R & D Systems, (Minneapolis, MN, USA) according to the manufacturer's instructions. Lipopolysaccharide (LPS), a positive control was used at a final concentration of $10 \mu\text{g mL}^{-1}$. The same controls used for the cytotoxicity experiments were employed. Cytokine concentrations were determined using the dual laser flow analyzer Luminescence 200 (Luminex Corp, Austin, TX, USA). Standard curves for each cytokine were plotted employing a 5-parameter logistic fit (5-PL). This procedure has been previously used and reported by our laboratory (Rodríguez-Cotto et al., 2014).

2.8 Statistical Analyses

Differences between individual groups were evaluated using the unpaired Student's *t* Test. The criterion for statistical significance was set at $p = 0.05$. Statistical analyses were performed using the Graph Pad InStat 3 software (GraphPad Software, Inc. La Jolla, CA). Analyses were based on 3 independent experiments per cell response.

3. Results and Discussion

The average mass of TPM collected during 30 min from the engine exhaust ranged from $455 \mu\text{g}$ (ethanol) to $1370 \mu\text{g}$ (biodiesel additive) (Table 1). As known that ethanol is the fuel that emits less particles. The metal content represented about 3-9 % of the total mass. Most of the elements analyzed (Ba, Cd, Co, Ga, Ni, Pb, Sb, Se, Ti and V) were not found in any kind of particles. The total metal content was much higher in soybean biodiesel than in fossil diesel or ethanol (Table 1). Some metals (Fe and K) were detected in higher concentration in soybean biodiesel compared to fossil diesel probably due to the process of collecting the soybean biodiesel at the steps where a catalyst was used. The metals Zn, Cr and Mn were also found in higher levels in soybean biodiesel. These same metals were also measured in the crude oils. A similar trend was observed for PAH where the sum of them was higher in soybean biodiesel than fossil diesel or ethanol (Table 1). The predominant PAH in soybean biodiesel and biodiesel additive were pyrene, benzo(b)fluoranthene and fluorene; in fossil diesel were phenanthrene, 1-methylnaphthalene and benzo(b)fluoranthene; and in ethanol were dibenz(a,h)anthracene, benzo(e)pyrene and benzo(b)fluoranthene. In all fuels benzo(a)pyrene was detected, with highest concentrations in pure soybean biodiesel. This compound is known as a potent carcinogen. Many of the PAH compounds related to benzene were found in particles obtained from soybean biodiesel and biodiesel additives. This is consistent with what other researchers have reported in the past (Agarwal et al., 2013).

3.1 Cytotoxicity

Biodiesel hexane extracts did not exert a noticeable adverse effect to BEAS-2B up to $75 \mu\text{g mL}^{-1}$ (Figure 1). This is in agreement with other research studies, which report that the most toxic fraction of biodiesel is the aqueous phase (Madden et al., 2011). Interestingly, a significant increase in cell viability was observed with both, B100A and B5 at $75 \mu\text{g mL}^{-1}$. Nevertheless, the induction was much higher for soybean biodiesel additive (B100A) compared to fossil diesel (B5). Pure soybean biodiesel extracts (B100) also promoted a significant increase in cell viability at $50 \mu\text{g mL}^{-1}$ indicating a cell proliferation enhancement by components of soybean biodiesel. Cell proliferation is an increase in cells number. Cell proliferation does not necessarily implicate a beneficial outcome because it is a phenomenon that characterizes tumor tissue growth throughout the entire organism. Carcinogens trigger cell proliferation and we have identified several carcinogens in biodiesel samples. Several studies present evidence that support the diesel exhaust-lung cancer hypothesis. Results from occupational studies with quantitative estimates of exposure have limitations, including weak and inconsistent exposure-response associations that could be explained by bias, confounding or chance, exposure misclassification, and often-inadequate latency. The weight of evidence is considered inadequate to confirm the diesel-lung cancer hypothesis (Gamble et al., 2012).

The use of DF indicates that metals are essential for that enhancement as evidenced by reducing the cell viability back to its normal levels in biodiesel with the additive exposure to B100A, $75 \mu\text{g mL}^{-1}$ (Figure 1). This effect is clearly seen with B100A because the sample containing the highest metal content of all fuel tested (Table 1) followed by B100, ethanol and finally B5. The cell proliferation effect in samples correlated well with the mass metal content in the extracts (Table 1), where $\text{B100A} > \text{B100} > \text{B5}$. Although no significant difference was observed in BEAS-2B treated with DF in B5 ($75 \mu\text{g mL}^{-1}$) or B100 ($50 \mu\text{g mL}^{-1}$) the cell proliferation effect was reduced indicating the involvement of metals in the response. The observed cellular growth reversal with DF disappears as metal concentrations increase (B100A, B100 at $100 \mu\text{g mL}^{-1}$) suggesting that there is an optimum metal concentration for it. This also suggests that organic constituents at higher concentration counteract the proliferation induced by metals.

Biodiesel samples extracted with acetone were not bare an effect on BEAS-2B up to the concentration of $75 \mu\text{g mL}^{-1}$ (Figure 2). The B100 exhibits a biphasic concentration distribution were cell growth proliferation is detected at concentrations of 10 and $75 \mu\text{g mL}^{-1}$. The cell proliferation was significantly reduced with the presence of DF, supporting again that the metal content is responsible for the cellular behavior. Samples B100, B5 as well as EtOH did not increase cell viability and there was no significant effect using DF. None of the soybean biodiesel or fossil diesel concentrations were toxic to BEAS-2B cells.

3.2 Cytokine Secretion

Cytokines are proteins, peptides or glycoproteins that are secreted by specific cells including lung epithelial cells. Cytokines function as signaling molecules that mediate and regulate immunity, inflammation and hematopoiesis. IL-6 is a pleiotropic cytokine that can stimulate B cells and promote T cell activation, growth and differentiation, participates in

inflammation, is one of the most important mediators of fever and stimulates energy mobilization in muscle and fatty tissues. IL-8 is a chemokine that recruits neutrophils which stimulate further inflammatory responses. IL-8 is known to be sensitive to alterations in redox potential and antioxidants responsible for neutrophil chemo-attraction and activation (Barnes, 2008; Hiraiwa and van Eeden, 2013). The ability of a cell to secrete cytokine can hamper its ability to respond to an environmental insult and hence the homeostatic balance in cells and tissues. The specific abundance, mixture and combination of metals can induce or alter cytokine secretions by cells. Cytokine release suppressed by metals can affect the signaling for a biological response (Rodríguez-Cotto et al., 2014). Both polar and nonpolar extracts were evaluated for cytokine secretion (IL-6 and IL-8) by BEAS-2B after 24 h extract exposure. IL-6 secretion did not significantly change under any type of exposure tested. However, an increase in IL-6 secretion was observed for B100 (50 $\mu\text{g mL}^{-1}$) when this sample was pre-incubated with DF but this increase was not statistically different due to high variability. This suggests that trace elements found in the biodiesel sample function as suppressor rather than as inducer of IL-6. This is an opposite effect of what is normally reported for particulate matter extracts and its metal contents. Particulate matter samples from Ro de Janeiro, Brazil were previously analyzed showing an increase and decrease in pro-inflammatory cytokines (Rodrguez-Cotto et al., 2014). The effect on IL-8 was similar to that of IL-6 with B100 (50 $\mu\text{g mL}^{-1}$) sample, however, it was more profound because the levels are significantly higher with DF (50 $\mu\text{mol L}^{-1}$) (Figure 3). More than a 4-fold increase in IL-8 secretion was observed with DF treatment, clearly demonstrating that heavy metals are responsible for the cytokine suppression effect. The polar fraction (acetone extract) generally showed an increase of IL-6 however due to the high variability this effect was not significantly different at $p < 0.05$ (Figure 4). It is also clear that this increase does not seem to be related to heavy metal concentration. Slight increases in IL-8 secretions are also evident but not significantly different from the controls. Previous research using BEAS-2B showed an increase in the secretion of IL-8 after 24 h exposures to B20 at 20 $\mu\text{g mL}^{-1}$, which is in the similar concentration range used in the present experiments (Fukagawa et al., 2013). However, tail pipe exhaust was found to be composed of more nonpolar organics than the B20 fuel. Here, it was noted the opposite, where more nonpolar organic composition was determined in biodiesel. This is probably due to differences in combustion and the type of engine used to collect exhaust combustion particles. There are few studies that show that biodiesel is not toxic (Agarwal et al., 2013) and/or a strong inducer of inflammation (Bnger et al., 2012). In fact some reports have shown that biodiesel exposure when compared to diesel enhances pulmonary inflammation through oxidative stress (Yanamala et al., 2013). This study however was performed in mice and considered histological morphology tissue changes, which suggest that biodiesel could cause more toxic effects than diesel particles.

4. Conclusion

Although recent findings on the possible adverse effects of biodiesel exhaust are contradictory, and result from the various variables such as fuel qualities, engine types and different operation conditions, we report that some of the soybean biodiesel presently being used in Brazil does not exhibit direct adverse effects on human lung cells nor they induce inflammatory response by means of cytokine release. These results however should not be

underestimated nor considered as an absolute positive outcome because an increase in cell proliferation does not necessary implicate a beneficial response. In fact cancer itself is considered a proliferative effect of cell growth. Exposure to environmental pollutants can in some way alter specific changes in cell molecular mechanisms and homeostasis that trigger this disease. We have also demonstrated that concurrent with this proliferative effect a parallel cytokines immune suppression response is present in lung epithelial cells. Whether this immune suppression could be related to cell proliferation itself or can play a role in maintaining internal cell balance needs to be investigated.

We demonstrate in this study that both of these cell effects are directly associated with metals exposure in the biodiesel used. Consequently these *in vitro* studies set the bases for evaluating Brazilian biodiesel and studying the effects with time in whole animals.

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References

- Agarwal AK, Gupta T, Dixit N, Shukla PC. Assessment of toxic potential of primary and secondary particulates/aerosols from biodiesel vis-a-vis mineral diesel fuelled engine. *Inhal Toxicol*. 2013; 25(6):325–332. <http://dx.doi.org/10.3109/08958378.2013.782515>. [PubMed: 23631768]
- Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest*. 2008; 118:3546–3556. [PubMed: 18982161]
- Becker S, Fenton MJ, Soukup JM. Involvement of microbial components and Toll-like receptors 2 and 4 in cytokine responses to air pollution particles. *Am J Respir Cell Mol Biol*. 2002; 27:611–618. [PubMed: 12397021]
- Bünger J, Krahl J, Schröder O, Schmidt L, Westphal GA. Potential hazards associated with combustion of bio-derived versus petroleum-derived diesel fuel. *Crit Rev Toxicol*. 2012; 42:732–750. <http://dx.doi.org/10.3109/10408444.2012.710194>. [PubMed: 22871157]
- da Cruz ACS, Leite MBN, Rodrigues LEA, Nascimento IA. Estimation of biodiesel cytotoxicity by using acid phosphatase as a biomarker of lysosomal integrity. *Bull Environ Contam Toxicol*. 2012; 89:219–224. <http://dx.doi.org/10.1007/s00128-012-0707-7>. [PubMed: 22717620]
- Diociaiuti M, Balduzzi M, De Berardis B, Cattani G, Stacchini G, Ziemacki G, Paoletti L. The two PM 2.5 (fine) and PM 2.5–10 (coarse) fractions: evidence of different biological activity. *Environ Res*. 2001; 86:254–262. [PubMed: 11453676]
- Farkas E, Bátka D, Kremper G, Pócsi I. Structure-based differences between the metal ion selectivity of two siderophores desferrioxamine B (DFB) and desferricoprogen (DFC): why DFC is much better Pb(II) sequestering agent than DFB? *J Inorg Biochem*. 2008; 102:1654–9. Epub 2008 Apr 1. DOI: 10.1016/j.jinorgbio.2008.03.006 [PubMed: 18486225]
- Fukagawa NK, Li M, Poynter ME, Palmer BC, Parker E, Gerlofs-Nijland ME, Totlandsdal AI, Tzamkiozis T, Leseman DLAC, Samaras Z, Per Schwarze ML, Ntziachristos L, Cassee FR. Cell Toxicity and Oxidative Potential of Engine Exhaust Particles: Impact of Using Particulate Filter or Biodiesel Fuel Blend. *Environ Sci Technol*. 2013; 47:5931–5938. <http://dx.doi.org/10.1021/es305330y>. [PubMed: 23597117]

- Gamble JF, Nicolich MJ, Boffetta P. Lung cancer and diesel exhaust: An updated critical review of the occupational epidemiology literature. *Critical Rev Toxicol*. 2012; 42:549–598. [PubMed: 22656672]
- Gerlofs-Nijland ME, Totlandsdal AI, Tzamkiozis T, Leseman DLAC, Samaras Z, Per Schwarze ML, Ntziachristos L, Cassee FR. Cell Toxicity and Oxidative Potential of Engine Exhaust Particles: Impact of Using Particulate Filter or Biodiesel Fuel Blend. *Environ. Sci Technol*. 2013; 47:5931–5938. <http://dx.doi.org/10.1021/es305330y>.
- Hawley, B.; L'Orange, C.; Olsen, DB.; Marchese, AJ.; Volckens, J. Oxidative stress and aromatic hydrocarbon response of human bronchial epithelial cells exposed to petro or biodiesel exhaust treated with a diesel particulate filter; *Tox Sci*. 2014. p. 1-35. <http://dx.doi.org/10.1093/toxsci/kfu147>
- Hiraiwa K, van Eeden SF. Contribution of lung macrophages to the inflammatory responses induced by exposure to air pollutants. *Mediat Inflamm* 619523. 2013:1–10.
- Madden, MC.; Bhavaraju, L.; Kodavanti, UP. Toxicology of Biodiesel Combustion. Products, Biodiesel- Quality, Emissions and By-Products. Montero, Gisela, editor. InTech; 2011. Available from: <http://www.intechopen.com/books/biodiesel-quality-emissions-and-byproducts/toxicology-of-biodiesel-combustion-products>
- Massone, CG.; Wagener, ALR.; Abreu, HM.; Gioda, A. Hydrocarbon concentration and source appraisal in atmospheric particulate matter (PM_{2.5}) of an urban tropical area; *Environ Sci Pollut Res Int* 2015. p. 1-7. <http://dx.doi.org/10.1007/s11356-015-4695-0>
- Mateus VL, Monteiro ILG, Rocha RCC, Saint Pierre TD, Gioda A. Study of the chemical composition of particulate matter from the Rio de Janeiro metropolitan region, Brazil, by inductively coupled plasma-mass spectrometry and optical emission spectrometry, *Spectrochim. Acta Part B Atomic Spectroscopy*. 2013; 86:131–136. <http://dx.doi.org/10.1016/j.sab.2013.03.003>.
- Mueller-Anneling L, Avol E, Peters J, Throne P. Ambient endotoxin concentrations in PM₁₀ from Southern California. *Environ Health Persp*. 2004; 112:583–588.
- Rodríguez Cotto RI, Ortiz Martínez MG, Rivera Ramírez E, Méndez LB, Dávila JC, Jiménez Vélez BD. African dust storms reaching Puerto Rican coast stimulate the secretion of IL-6 and IL-8 and cause cytotoxicity to human bronchial epithelial cells (BEAS-2B). *Health*. 2013; 5(10A2):14–28. <http://dx.doi.org/10.4236/health.2013.510A2003>. [PubMed: 25002916]
- Rodríguez-Cotto RI, Ortiz-Martínez MG, RiveraRamírez E, Mateus VL, Amaral BS, Jiménez-Vélez BD, Gioda A. Particle Pollution in Rio de Janeiro, Brazil: Increase and Decrease of Pro-inflammatory Cytokines IL-6 and IL-8. *Environ Pollut*. 2014; 194:112–120. <http://dx.doi.org/10.1016/j.envpol.2014.07.010>. [PubMed: 25106047]
- Steiner S, Czerwinski J, Comte P, Popovicheva O, Kireeva E, Müller L, Heeb N, Mayer M, Fink A, Rothen-Rutishauser B. Comparison of the toxicity of diesel exhaust produced by bio- and fossil diesel combustion in human lung cells in vitro. *Atmos Environ*. 2013; 81:380–388.
- Swaran JS, Pachauri V. Chelation in Metal Intoxication. *Int J Environ Res Public Health*. 2010; 7:2745–2788. DOI: 10.3390/ijerph7072745 [PubMed: 20717537]
- Traviss N, Li M, Lombard M, Thelen BA, Palmer BC, Pointer ME, Mossman BT, Holmén BA, Fukagawa NK. Petrodiesel and waste grease biodiesel (B20) emission particles at a rural recycling center: characterization and effects on lung epithelial cells and macrophages. *Air Qual Atmos Hlth*. 2014; 7(1):59–70. <http://dx.doi.org/10.1007/s11869-013-0231-x>.
- Vandebriel R, Cransveld CC, Crommelin D, Diamant Z, Glazenburg B, Joos G, van Loveren H. Respiratory sensitization: advances in assessing the risk of respiratory inflammation and irritation. *Toxicol in Vitro*. 2011; 25:1251–1258. [PubMed: 21570460]
- Yanamala N, Hatfield MK, Farcas MT, Schwegler-Berry D, Hummer JA, Shurin MR, Birch ME, Gutkin DW, Kisin E, Kagan VE, Bugariski AD, Shvedova AA. Biodiesel versus diesel exposure: Enhanced pulmonary inflammation, oxidative stress, and differential morphological changes in the mouse lung. *Toxicol Appl Pharmacol*. 2013; 272:373–383. <http://dx.doi.org/10.1016/j.taap.2013.07.006>. [PubMed: 23886933]

Figure 1.

Cytotoxicity of BEAS-2B to pure soybean biodiesel (B100-50 to $75 \mu\text{g mL}^{-1}$), soybean biodiesel additive (B100A-50 to $100 \mu\text{g mL}^{-1}$), and fossil diesel (B5-50 to $75 \mu\text{g mL}^{-1}$) extracted with hexane (nonpolar fraction). Cells were treated with the extracts in the presence or absence of $50 \mu\text{mol L}^{-1}$ deferoxamine (DF). Triton X (+ CNT) was used as a positive control at a concentration of $25 \mu\text{g mL}^{-1}$. A cut off value of 80 % was considered as cytotoxic. Bars represent mean % cell viability $\pm$ SEM, *** $p < 0.001$, * $p < 0.05$ compared to medium or to the adjacent treatment when upper solid line is shown ($n = 3$).

Figure 2.

Cytotoxicity of BEAS-2B to pure soybean biodiesel (B100), soybean biodiesel additive (B100A), fossil diesel (B5) and ethanol (EtOH) at the concentration range of 10-75 µg mL⁻¹. Cells were treated with the extracts in the presence and absence of 50 µmol L⁻¹ deferoxamine (DF). Triton X (+ CNT) was used as a positive control at a concentration of 25 µg mL⁻¹. A cut off value of 80 % was considered cytotoxic. Bars represent mean % cell viability ± SEM, ***p < 0.001, **p < 0.01 compared to medium or to the adjacent bar treatment (solid line), n = 3.

Figure 3.

Cytokine A) IL-6 and B) IL-8 secretion by BEAS-2B exposed for 24 h to pure soybean biodiesel (B100-50 $\mu\text{g mL}^{-1}$), soybean biodiesel additive (B100A-75 $\mu\text{g mL}^{-1}$) and fossil diesel (B5-75 $\mu\text{g mL}^{-1}$) extracted with hexane. Lipopolysaccharide (LPS at 10 $\mu\text{g mL}^{-1}$) was used as positive control. Bars represent mean cytokine concentration $\pm$ SEM, * $p < 0.05$ compared to DMSO, $n = 3$.

Figure 4. Cytokine A) IL-6 and B) IL-8 secretion by BEAS-2B exposed to pure soybean biodiesel (B100), soybean biodiesel additive (B100A) and fossil diesel (B5) extracted with acetone at $75\mu\text{g mL}^{-1}$ for 24 h. Lipopolysaccharide (LPS at $10\mu\text{g mL}^{-1}$) was used as positive control. Bars represent mean cytokine concentration $\pm$ SEM, $n = 3$.

Table 1

Content of polycyclic aromatic hydrocarbons (ng), metals (µg) and total particulate matter (µg) measured in the fuel emissions.

PAH	B100 (pure soybean biodiesel)	B100A (soybean biodiesel with BHT additive)	B5 (fossil diesel with 5% biodiesel)	EtOH (91.06 % ethanol, 7.9 % NTHF and 0.99 % castor oil)
1-methylnaphtalene	Nd	Nd	41.45	4.95
2-methylnaphtalene	6.15	Nd	119.33	9.87
Acenaphtalene	7.13	7.23	Nd	1.99
Acenaphthylene	7.02	Nd	6.83	2.05
Anthracene	11.77	9.56	16.15	2.36
Benzo(a)anthracene	179.33	100.12	33.91	9.82
Benzo(a)pyrene	53.35	34.64	24.68	29.78
Benzo(b)fluoranthene	454.13	274.36	97.37	31.25
Benzo(e)pyrene	222.72	114.77	40.35	33.22
Benzo(ghi)perylene	183.81	92.74	38.59	2.73
Benzo(k)fluoranthene	195.92	155.43	25.03	3.15
Chrysene	219.57	144.57	50.11	13.35
Dibenz(a,h)anthracene	6.81	Nd	Nd	38.68
Dibenzothiophene	10.00	5.56	10.03	4.20
Fluoranthene	10.21	12.30	17.44	6.11
Fluorene	408.41	309.60	68.24	13.87
Indeno(1,2,3-cd)pyrene	128.77	81.13	30.20	11.33
Naphthalene	5.08	4.51	50.69	7.10
Perylene	4.40	Nd	1.82	4.69
Phenanthrene	157.51	94.10	112.08	23.05
Pyrene	521.70	441.01	60.05	30.68
Total PAH	2,793.78	1,881.63	844.34	284.24
Metals				
Al	2.45	1.35	2.60	2.10
Ca	19.80	9.40	12.35	10.00
Cr	0.90	Nd	0.45	Nd
Cu	0.55	0.40	0.40	1.55
Fe	36.25	39.95	Nd	3.00
K	14.80	20.10	0.25	Nd
Mg	2.50	1.95	2.05	1.50
Mn	0.15	0.10	0.05	0.05
Na	1.50	17.30	0.90	23.60
Zn	3.40	1.80	1.00	2.50
Total Metals	82.60	92.35	20.05	44.30
Total Particulate Matter	1160	1370	793	455

Nd: indicates that the measurement is below the detection limit.

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