NISEB Journal Vol. 17, No. 4. December, 2017 1595-6938/2017
Printed in Nigeria (2017) Society for Experimental Biology of Nigeria
http://www.nisebjournal.org

Toxicological Evaluation of Biodiesel Emission Particles (BEP) Using Rat Models

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Abstract
The present study is an attempt to elucidate the effect of biodiesel emission particles (BEP) on some antioxidant enzymes of selected tissues of rats. Rats were exposed to emission particles (EP) of biodiesel blended with fuel diesel at 100BD, 75BD, 50BD and 25BD as well as fuel diesel (FD) over a period of ten days. Enzyme assays were conducted for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and reduced glutathione (GSH) in the liver, lungs, brain and serum of rats. Lungs CAT activity of Control is 229% higher than that of FD and789% that of 100BD. There existed no significant difference ($p>0.05$) in the brain CAT activity among all treatment groups. Serum CAT activity of Control group was significantly higher ($p<0.05$) relative to other groups of rats and about 2 folds that of rats in FD group. Data obtained from this study shows that the BEP is capable of inducing oxidative stress in tissues of rats by a mechanism where superoxides, lipid hydroperoxides and hydrogen peroxides are generated which in turn depletes total antioxidant capacity (TAC) of the rat.

Key words: Biodiesel, emission particles, diesel, antioxidants, enzymes

Introduction
Dwindling oil reserves, expanding capitalization, increasing fuel prices, socio-economic and environmental problems have heightened the interest in renewable and affordable energy sources. Renewable energy is energy from non-vanishing natural resources, such as, sunlight, wind, water, tide/wave, geothermal and biomass. Biodiesel is seen as a viable alternative fuel to petroleum-based diesel and commonly referred to as an energy carrier; it is also classified as a type of modified or enhanced fuel. (1). Biodiesel refers to the pure fuel before blending with diesel fuel occurs; it is also considered as neat biodiesel.

The toxicology of combusted biodiesel is an emerging field. Much of the current knowledge about biological responses and health effects stems from studies of exposures to incompletely combusted other fuel sources (typically petroleum diesel, gasoline, and wood). The ultimate aim of toxicology studies of biodiesel emission particles (BEP) is to identify possible health effects induced by exposure of both the general population as well as sensitive or susceptible populations. Notable is the fact that possible health effects may take years of exposure to discern, e.g., lung cancer, fibrosis, emphysema, etc., mitigation of the exposure and/or effects may be too late for an individual (2). Consequently, markers and biological responses believed to be an early step leading to a clinical disease are measured as a surrogate of the health effect (3). In this study, activities of antioxidant enzymes are used as biomarkers that will ultimately lead to a disease induced by exposure to a pollutant.

Materials and Methods
Reagents and solvents were of analytical grade and are products of British Drug House, Poole, England. Vegetable oil was purchased at the local market in Effurun, Delta State. Nigeria.

Preparation of Bio-diesel from Vegetable oil

Biodiesel was prepared from vegetable oil in accordance with the method described by Aremu *et al* (4).

Biodiesel Blend and the Experimental Rat Treatment

The Biodiesel from vegetable oil was blended with fuel diesel and grouped as follows:
Blend 1: 100% fuel diesel (100FD)
Blend 2: 100% biodiesel (100BD)
Blend 3: 75% biodiesel and 25% fuel diesel (75BD)
Blend 4: 50% biodiesel and 50% fuel diesel (50BD)
Blend 5: 25% biodiesel and 75% fuel diesel (25BD)

Eighteen (18) albino rats were obtained from an animal house of Department of Anatomy University of Benin, Benin City. Nigeria and were allowed to acclimatized for fourteen (14) days prior to the commencement of the experiment. The experimental rats were grouped into six (6) of three (3) rats in each group:
Group 1: served as control and the rats were not exposed to any fuel smoke/flame
Group 2: rats exposed to FD 1minute per day for 10days
Group 3: rats exposed to 100BD 1minute per day for 10days

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Group 4: rats exposed to 75BD 1minute per day for 10days
Group 5: rats exposed to 50BD 1minute per day for 10days
Group 6: rats exposed to 25BD 1minute per day for 10days
The experimental rats were fed *ad libitum* with commercial rat chow throughout the experiment period.
A glass chamber was constructed in the Department of Mechanical Engineering FUPRE which was used to enclose the experimental rats while they were being exposed to the BEP 1 minute daily for 10 days. After which the animals were sacrificed at the end of the experiment.
The rats were anaesthetized by placing them in a jar containing cotton wool soaked with chloroform before being sacrificed by jugular puncture. And were quickly dissected and the whole liver, lungs, brain were excised, freed of fat, blotted with clean tissue paper and weighed into a beaker containing ice cold 0.25M sucrose solution. The blood was obtained through cardiac puncture. A portion of the blood was collected in heparinised bottles and others in non-heparinised bottles. Some blood samples were thereafter centrifuged at 3,500 rpm for about 15 min using refrigerated centrifuge RC650s and the serum samples obtained were preserved at-8^{o}C until required for analyses. A portion of each organ was homogenized for enzyme assays. The diluted homogenates were stored at temperature of -8^{o}C until required for use.
The GSH concentration in the tissues of experimental rats was determined following the method described by Jollow *et al,* (5). The SOD activity of the tissues of experimental animals was determined following the method described by Misra and Fridovich (6). The catalase activity of the tissue homogenate obtained from the experimental animals was determined following the method described by Sinha (7). The GPx concentration in the tissues of experimental rats was determined following the method described by Jollow *et al,* (5).

Statistical Analyses

All numerical results were obtained from the three (6) groups (control and treated). Data obtained were presented as mean±SEM and subjected to statistical analysis using a one way analysis of variance (ANOVA) by employing the method of Steel and Torrie (8). Significant difference between the treatment means was determined at 95% confidence level using Duncan's Multiple range test (9).

Results

The specific activity of superoxide dismutase (SOD) of tissues of rats exposed to combustible flames of biodiesel over a period of ten days is shown in Table 1. Generally, specific activity of SOD of the liver of control rats was found to be significantly higher ($p<0.05$) than those of other rat groups. However, no significant difference ($p>0.05$) existed between the SOD activity of liver of rats in 100BD group relative to the control. It was found also, that the SOD activity of liver of 100BD group of rats is not significantly different ($p>0.05$) from that of rats in 75BD group. The SOD of lungs of rats in control group is significantly higher ($p<0.05$) than that of any other group. It was found to be about 3folds the SOD activity of the SOD of lungs of rats in other groups. Although, the activity of SOD of brain of rats in control group was significantly higher ($p<0.05$) than of groups of rats, yet there existed no significant difference ($p>0.05$) among the other groups of rats. Similarly, serum SOD activity of control rats was significantly higher than that of any other group. Besides the SOD activity of rats in FD group that was significantly lower ($p<0.05$) than that of other test groups, the SOD activity among 100BD, 75BD, 50BD and 25BD groups of rats.

Table 1: The specific activity of superoxide dismutase (SOD) of tissues of rats exposed to combustible flames of biodiesel over a period of ten days

Tissues	**Control**	**FD**	**100BD**	**75BD**	**50BD**	**25BD**
Liver	2.32 ± 0.13^{a}	1.89 ± 0.10^{b}	2.22 ± 0.12^{ac}	2.10 ± 0.09^{c}	1.97 ± 0.09^{bc}	1.91 ± 0.07^{bc}
Lungs	1.7 ± 0.02^{a}	0.50 ± 0.01^{b}	0.53 ± 0.01^{c}	0.68 ± 0.01^{d}	0.60 ± 0.01^{e}	0.56 ± 0.01^{f}
Brain	7.22 ± 0.67^{a}	5.54 ± 0.55^{b}	6.04 ± 0.58^{b}	5.88 ± 0.49^{b}	5.75 ± 0.45^{b}	5.70 ± 0.50^{b}
Serum	0.12 ± 0.01^{a}	0.06 ± 0.01^{b}	0.09 ± 0.01^{c}	0.09 ± 0.01^{c}	0.07 ± 0.01^{c}	0.07 ± 0.01^{c}

Values on the same row bearing different superscripts are significantly different ($P<0.05$). Tabulated data are means of three (3) determinations ± SEM.

Table 2: The specific activity of Catalase (CAT) of tissues of rats exposed to combustible flames of biodiesel over a period of ten days

Tissues	Control	FD	100BD	75BD	50BD	25BD
Liver	868.0±65.7^{a}	507±56.5^{b}	871±60.2^{a}	800±59.8^{ab}	736±60.5^{bc}	628±57.3^{c}
Lungs	3.2±0.50^{a}	0.98±0.45^{b}	0.36±0.12^{c}	1.18±0.38^{bd}	1.35±0.21^{d}	1.05±0.22^{b}
Brain	5.77±0.76^{a}	4.87±0.64^{a}	5.35±0.52^{a}	5.05±0.49^{a}	5.12±0.53^{a}	5.00±0.45^{a}
Serum	1.25±0.04^{a}	0.67±0.01^{b}	0.88±0.02^{c}	1.02±0.02^{d}	0.96±0.01^{e}	0.89±0.01^{c}

Values on the same row bearing different superscripts are significantly different (P<0.05). Tabulated data are means of three (3) determinations ± SEM.

Table 2 shows the specific activity of catalase (CAT) of tissues of rats exposed to combustible flames of biodiesel over a period of ten days. Specific activity of CAT of liver of 100BD and 75BD rats was not significantly different (p>0.05) from that of the control while CAT activity of liver of rats in FD, 50BD and 25BD groups was significantly lower (p<0.05) relative to the control. Conversely, in the lungs, CAT activity of control rats was significantly higher (p<0.05) relative to other groups. Lungs CAT activity of Control is 229% higher than that of FD and789% that of 100BD in particular. Interestingly, there existed no significant difference (p>0.05) in the brain CAT activity among all treatment groups. Serum CAT activity of Control group was significantly higher (p<0.05) relative to other groups of rats and about 2 folds that of rats in FD group in particular.

Glutathione peroxidase (GPX) activity of tissues of rats exposed to combustible flames of biodiesel over a period of ten days is presented in Table 3. Generally, GPX activity of tissues of Control rats was significantly higher (p<0.05) relative to other treatment groups. Brain GPX activity, among other treatment groups, aside the Control, was not significantly different (p>0.05). In the there was no significant difference between activity of GPX of 100BD and 75BD, as well as 50BD and 25BD rats respectively.

Table 3: Glutathione peroxidase (GPX) activity of tissues of rats exposed to combustible flames of biodiesel over a period of ten days

Tissues	Control	FD	100BD	75BD	50BD	25BD
Liver	118.67±6.72^{a}	67.45±5.66^{b}	87.33±6.18^{c}	89.46±5.99^{c}	74.67±6.10^{d}	70.16±5.98^{d}
Lungs	60.23±2.34^{a}	35.77±3.17^{b}	54.13±2.81^{c}	47.66±2.18^{d}	42.18±3.05^{e}	41.47±2.88^{e}
Brain	23.18±1.36^{a}	16.46±1.81^{b}	18.96±1.04^{b}	18.32±1.50^{b}	17.48±1.02^{b}	17.02±1.11^{b}
Serum	1.80±0.03^{a}	0.96±0.01^{b}	1.12±0.03^{c}	1.00±0.02^{d}	0.98±0.01b^{d}	0.99±0.01^{bd}

Values on the same row bearing different superscripts are significantly different (P<0.05). Tabulated data are means of three (3) determinations ± SEM.

Table 4: The concentration of reduced glutathione of tissues of rats exposed to combustible flames over a period of ten days

Tissues	Control	FD	100BD	75BD	50BD	25BD
Liver	22.70±1.13^{a}	13.78±1.00^{b}	14.39±0.98^{b}	16.74±1.05^{c}	18.86±0.68^{d}	15.12±0.88^{bc}
Lungs	2.65±0.05^{a}	0.56±0.01^{b}	0.34±0.01^{c}	1.10±0.04^{d}	1.16±0.06^{d}	0.78±0.02^{e}
Brain	3.01±0.28^{a}	1.18±0.22^{b}	2.01±0.28^{c}	2.52±0.25^{c}	2.64±0.18^{c}	1.58±0.16^{d}
Serumμmol/l	2.18±0.58^{a}	0.67±0.02^{b}	0.89±0.02^{c}	1.47±0.12^{d}	1.88±0.11^{e}	1.30±0.09^{f}

Values on the same row bearing different superscripts are significantly different (P<0.05). Tabulated data are means of three (3) determinations ± SEM.

The concentration of reduced glutathione of tissues of rats exposed to combustible flames over a period of ten days is presented in Table 4 GSH content of liver of rats in FD, 100BD and 25BD groups was not significantly different ($p>0.05$), similarly the GSH content of liver of rats in 75BD and 25BD was also not significantly different ($p>0.05$). Conversely, liver GSH of Control rats was significantly higher ($p<0.05$) relative to that of the other treatment groups. In the lungs, GSH of rats in Control group was 2 folds that of rats in 75Bd and 50BD groups, and about 4 folds that of rats in FD and 25BD groups. Brain GSH of 100BD, 75BD and 50BD was not significantly different ($p>0.05$). GSH content of brain of Control rats was significantly higher ($p<0.05$) relative to other treatment groups, it was about 200% that of brain of FD and 25BD group of rats. In the serum however, GSH content was significantly different ($p<0.05$) among all treatment groups.

Discussion

Biodiesel is one of the most promising alternative diesel fuels. It is the only alternative fuel to have fully completed the health effects testing requirements of the Clean Air Act. It may be made from a variety of different vegetable oils or animal fats. It has slightly lower fuel density than regular diesel fuel, so more fuel is required to achieve the same amount of power. Compared to regular diesel fuel, biodiesel also has higher viscosity, affecting spray formation; higher pour point and cloud points, limiting winter operation; lower oxidative stability, shortening storage life; and higher organic carbon emissions. Emissions of total PM, CO, and HC are reduced by using biodiesel fuel (10, 11), while those of particle-bound volatile organic material (organic carbon) are increased (12, 13), and those of NO*x* are slightly higher (11, 14) However, recent work suggests that NO*x* emissions either increase or decrease slightly depending upon the vehicle and test condition (14). This study examined the effect of biodiesel flame on oxidative enzymes of selected tissues of rats. Antioxidants serve as a means of self-defense designed by nature to decompose peroxides, inactivate metals, scavenge free radicals and hinder lipid peroxidation. These antioxidants can be enzymes (SOD, catalase, glutathione peroxidase and glutathione-s-transferase) and non-enzyme (glutathione, ascorbic acid, ubiquinones, and α-tocopherol etc). Abnormal levels of these antioxidants in the body portend a condition of oxidative stress (15).

Superoxide dismutase (SOD) enzyme destroys the superoxide radical; however, as a result of that it creates hydrogen peroxide, which also has high toxic properties. It has been reported as one of the most important antioxidant defense enzyme that scavenge superoxide anion by converting to hydrogen peroxide thus diminishing the toxic effect caused by this radical (16). The reduced SOD activities of tissues of rats exposed to flame of biodiesel, observed in this study (Table1), may be due to presence of high levels of superoxides generated by the flames of biodiesel which were being converted to hydrogen peroxides by SOD thus limiting the toxic effect.

Results of catalase activity (Table 2) lend credence to the results of SOD as it also decreased in tissues of rats exposed to biodiesel flames. Catalase (CAT) is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS). Catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen per second (15). The observed result was perhaps, due to the elevated level of hydrogen peroxide generated partly from SOD activity and partly from tissue response to flames of biodiesel.

GPx catalyzes the glutathione-dependent reduction of lipid hydroperoxides and hydrogen peroxide for detoxification (17). GPx of tissues of rats (Table 3) decreased when exposed to flame of biodiesel; this result suggests that flames of biodiesel induced formation of both lipid hydroperoxides and hydrogen peroxides which in turn decreased GPx activity. To buttress the submission, level of reduced glutathione (GSH) of tissues of rats exposed to biodiesel flame showed a decline in GSH (Table 4). GSH is an antioxidant, preventing damage to important cellular components caused by reactive oxygen species (ROS) such as free radicals and peroxides. Glutathione, a major nonprotein thiol in living organisms, which plays a central role in coordinating the body's antioxidant defense processes. Excessive peroxidation causes increased glutathione consumption. Reduced thiols have long been reported to be essential for recycling of antioxidants like vitamin E and vitamin C. GSH plays a very important role in the detoxification of xenobiotics. *In vitro* examinations proved that the free thiol group of glutathione reacts with xenobiotics to form conjugates. From the foregoing, flame of biodiesel probably induces glutathione-dependent peroxidation thus causing depletion of glutathione-dependent antioxidants as well.

Data obtained from this study shows that the particulate matter contained in biodiesel flame is capable of inducing oxidative stress in tissues of rats by a mechanism where superoxides, lipid hydroperoxides and hydrogen peroxides are generated which in turn depletes total antioxidant capacity (TAC) of the rat. Despite the certificate of Clean Air Act endorsed for biodiesel after the completion of health effects testing requirements, this study has shown that exposure to flames of biodiesel could pose a risk to public health.

Acknowledgement
The authors wish to sincerely appreciate the contribution of Mr Frank Onwugbolu of Mechanical Engineering Workshop, Federal University of Petroleum Resources Effurun who helped to construct the glass chamber used in this study.

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