Original Research Article

Biodiesel causes Oxidative Damage in tissues of *Clarias gariepinus*

Abstract

Aim: Alternative fuels have become more prominent today because of environmental concerns. Due to the increase in the use of alternative fuels, toxicology studies have become imperative to determine whether alternative fuels will affect the biochemistry of aquatic organisms.
Study Design: In this study, biodiesel in different concentrations (0.0, 0.1, 0.25 %v/v) was introduced into water samples of same volume containing species of C*larias gariepinus* (African cat fish).
Place and Duration of Study: This study was carried out in the Department of Environmental Science, Federal University of Petroleum Resources, Effurun, Nigeria from April to October 2014.
Methodology: The 3 groups of fish placed in (0.0 0.1, 0.25)%v/v biodiesel-contaminated water were sacrificed after 30hours and enzymic and non-enzymic antioxidants (GSH, SOD, CAT, and MDA) as well as haematological properties were analyzed.
Results: Specific activity of SOD was found to be 8.55±0.89, 6.25±0.45 and 6.22±0.55 in the kidney of Control, 0.1%v/v and 0.25%v/v fish respectively. Similarly, specific activity of catalase was found to be 18.24±1.89, 15.30±0.76 and 13.39±1.27 in the gills of Control, 0.1%v/v and 0.25%v/v fish respectively. Conversely, the haematological property of Control is not significantly different from those of 0.1%v/v and 0.25%v/v fish. Results from this study showed significant decrease in the antioxidant status of cat fish from biodiesel contaminated water, however, haematological properties of the fish were not affected. This study revealed that biodiesel from palm kernel oil poses threat to aquatic life forms.

Keywords: Biodiesel, oxidative stress, tissue, palm kernel oil, aquatic, haematology

Introduction

The increased demand for alternative energy sources has created interest in biodiesel and biodiesel blends; biodiesel is promoted as a diesel substitute that is safer, produces less harmful combustion emissions, and biodegrades more easily. Like diesel spills, biodiesel can have deleterious effects on the aquatic environments [1]. Fish live in very intimate contact with their environment, and are therefore very susceptible to physical and chemical changes which may be reflected in their blood components [2-3].
Cellular antioxidant defense systems in biological systems are impaired when exposed to environmental pollutants, but the levels of antioxidants in living organisms can increase in order to restore the imbalance caused by oxidative damage. Levels of antioxidant enzymes can be used as an indicator of the antioxidant status of the organism and can serve as biomarkers of oxidative stress [4]. When antioxidant defenses are impaired or overcome, oxidative stress may produce DNA damage, enzymatic inactivation and peroxidation of cell constituents, especially lipid peroxidation [5]. Toxicity biomarkers, such as malondialdehyde (MDA), have been also proposed to reflect the oxidative status of exposed species [6]. MDA is used as marker of oxidation of membrane phospholipids through lipid peroxidation. An increase in MDA levels in organisms can be related to degradation of an environmental site by decreasing the water quality

[7]. The level of antioxidant enzymes have been extensively used as an early warning indicator of lake pollution [8].

Enzymatic and non-enzymatic antioxidants serve as an important biological defense against environmental pollutants. Studies on the oxidative indices of catfish associated with biodiesel are very scanty, literature on the impacts of other toxicants or effluent abound. Thus, the purpose of this study is to evaluate the effect of biodiesel produced from PKO on enzymic and non-enzymic antioxidant of some selected tissues of fish using African cat fish (*Clarius gariepinus*) as a model.

Materials and Methods

Reagents and solvents were of analytical grade and are products of British Drug House, Poole, England.

Perm Kernel Oil (PKO)

Palm kernel oil was purchased at the local market in Effurun, Nigeria. 100g PKO was used for the transesterification process. The ethanol used (99% pure) is an analytical grade with boiling point of 78^{o}C; while the NaOH used was also an analytical grade product of Aldrich Chemicals, England. The blender used was a Dry and Wet mill Blender with a clear glass (1,250 cc capacity) containers and stainless steel cutting blades. Other major materials used include scales, translucent white plastic container with bung and screw-on cap, funnels, PET bottles and thermometer.

Preparation of Bio-diesel from PKO

Biodiesel was prepared from PKO in accordance with the method described by Alamu *et al* [9].

Experimental Water and Fish Treatment

The Biodiesel from PKO was diluted with borehole water to obtain 0.25 and 0.1 %v/v. Twenty-four healthy juvenile catfish (*Clarias gariepinus*) were obtained from a commercial fish pond at Ekpan in Delta State, Nigeria and acclimatized for ten days prior to the commencement of the experiment. The catfish were grouped into three (3) of eight catfish and were kept in 30L plastic aquaria. Group A served as control and the catfish here were cultured in borehole water while those in Groups B and C were exposed to the different mixtures (0.1%v/v and 0.25% v/v respectively) of Biodiesel from PKO. The catfish were fed *ad libitum* with commercial fish meal for 30hrs during which the experiment lasted.

The cat fish were sacrificed at the end of the experiment and were quickly dissected and the whole liver, kidney, brain and heart were excised, freed of fat, blotted with clean tissue paper and weighed. A portion of each organ was homogenized for biochemical studies and enzyme assays. The blood was obtained through cardiac puncture. A portion of the blood was collected in heparinised bottles and others in nonheparinised 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 at81C until required for analyses. Haemoglobin concentration of the blood of experimental animals was determined following the method described by Mitruka

and Rawnsley [10]. The RBC and WBC was done by the method of manual counting, PCV by Microhaematocrit method, described by Muthayya [11]. Other haematological parameters were determined as described by Tiez [12]. The protein content in the tissue homogenates were determined using the Biuret method of Gornal *et al*. [13]. Cupric ions in alkaline solution form a purple coloured complex with any compound containing repeated-CONH-links such as proteins. The colour intensity which is a measure of the protein content of a sample is measured spectrophotometrically at 540nm. MDA determination was based on method described by Bird et al. [14]. MDA reacts with thiobarbituric acid to give a red complex which is measured spectrophotometrically at 535nm. The method described by Jollow *et al* [15] was used to determine reduced glutathione (GSH) concentration. The absorbance was read at 412nm. Catalase activity was determined according to the method of Sinha [16]. The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2 with the formation of perchloric acid as an unstable intermediate. The chromic acetate was then measured spectrophotometrically at 570nm. The activity of superoxide dismutase (SOD) was determined by the method of Misra and Fridovich [17]. This method is based on the ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2. The absorbance was measured at 480nm.

Statistical Analyses

All numerical results were obtained from the three (3) 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 [18]. Significant difference between the treatment means was determined at 95% confidence level using Duncan's Multiple range test [19].

Results

Haematological properties of *C. gariepinus* cultivated in contaminated water are presented in Table 1. Generally, result from this experiment showed no significant difference ($p>0.05$) in the haematological parameters among the three (3) groups of *C. gariepinus*. It was further observed that the value of Eosiniphils (%) for the three groups of catfish is zero (0).

Table 1: Haematological properties of *Clariasgariepinus* cultivated in water contaminated with bio-fuel from PKO

Haematological parameters	Group A	Group B	Group C
RBC ($x10^6/mm^3$)	2.71±0.10[a]	2.69±0.12[a]	2.72±0.31[a]
Hb (g/dL)	5.76±0.54[a]	5.23±0.56[a]	5.56±0.46[a]
MCV (μ^3)	58.99±2.45[a]	56.78±2.34[a]	56.39±2.07[a]
MCH (µµg)	14.30±0.86[a]	14.34±0.73[a]	13.97±1.06[a]
MCHC (%)	16.74±1.11[a]	15.98±0.99[a]	16.58±1.02[a]
PCV (%)	21.32±1.23[a]	21.56±1.21[a]	21.12±1.48[a]
WBC ($x10^3/mm^3$)	25.75±1.53[a]	26.01±1.13[a]	27.03±1.72[a]
Neutrophils (%)	3.48±0.34[a]	3.25±0.52[a]	3.33±0.65[a]
Eosinophils (%)	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
Basophils (%)	0.34±0.01[a]	0.32±0.01[a]	0.32±0.01[a]

Lymphocytes (%)	23.63±1.78 [a]	23.56±1.67 [a]	23.95±2.00 [a]
Monocytes (%)	13.20±1.02 [a]	13.22±1.33 [a]	13.09±1.51 [a]

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

The GSH concentrations of tissues of African cat fish *(Clarias gariepinus)* cultivated in water contaminated with biodiesel produced from PKO is presented in Table 2. Generally, no significant difference (P>0.05) was found in the GSH content of serum of the experimental fish. Conversely, the GSH content of Liver of group C fish was significantly lower than that of group A, while the GSH content of Liver of group B, was not significantly different (P>0.05) from that of groups A and C.

Table 2: Concentration of reduced glutathione (µg/mg tissue) of liver and blood of *Clarias gariepinus* cultivated in water contaminated with bio-fuel from PKO.

Group	Liver	Blood
A	10.23±1.24[a]	18.23±1.12[a]
B	9.21±1.43[ab]	18.64±1.23[a]
C	7.56±1.11[b]	18.52±1.19[a]

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

Table 3 shows activity of SOD of *Clarias gariepinus* cultivated in biodiesel contaminated water. In contrast to the activity of SOD of the liver and brain of experimental fish, the activity of SOD of kidney and gill of fish in Groups B and C was found to be significantly lower than that of the control fish (p<0.05).

Table 3: Specific activity of superoxide dismutase (Unit/mg protein) of selected tissues of *Clarias gariepinus* cultivated in water contaminated with bio-fuel from PKO.

Group	Brain	Liver	Kidney	Gill
A	3.21±0.45[a]	7.86±1.43[a]	8.55±0.89[a]	5.23±0.98[a]
B	2.98±0.36[a]	6.42±1.29[a]	6.25±0.45[b]	3.24±0.87[b]
C	3.02±0.68[a]	6.13±1.11[a]	6.22±0.55[b]	2.89±0.89[b]

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

The activity of catalase in tissue of fish *clarias gariepinus* introduced into water contaminated with biodiesel produced from PKO is presented in Table 4. Catalase activity of the liver, kidney and gills of *clarias gariepinus* introduced into PKO biodiesel contaminated water was significantly lower (P>0.05) than that of control fish. Conversely, no significant difference was found in the activity of catalase of brain of test fish relative to the control (p<0.05).

Table 4: Specific activity of catalase (µmole of H_2O_2 decomposed/min/mg protein) of selected tissues of *Clarias gariepinus* cultivated in water contaminated with bio-fuel from PKO

Group	Brain	Liver	Kidney	Gill
A	1.58±0.18a	15.96±1.00a	18.54±2.34a	18.24±1.89a
B	1.46±0.09a	13.22±0.75ab	13.77±1.69b	15.30±0.76b
C	1.39±0.11a	12.56±0.92b	11.78±1.95b	13.39±1.27c

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

The MDA concentrations of tissues of Africa cat fish (*Clarias gariepinus*) introduced into water contaminated with biodiesel produced from PKO are presented in Table 5. The MDA concentrations of serum and brain of test fish were not significantly different (P>0.05) that of control. Conversely, levels of MDA in the liver, kidney and gills of fish introduced into the PKO biodiesel contaminated water of different concentration (group B & C) were significantly lower (P<0.05) than that of control fish.

Table 5: Concentration of malondialdehyde (nmol/mg tissue) of selected tissues of *Clarias gariepinus* cultivated in water contaminated with bio-fuel from PKO

Group	Brain	Liver	Kidney	Gill	Serum
A	0.37 ± 0.01^{a}	0.21 ± 0.01^{a}	0.23 ± 0.02^{a}	0.10 ± 0.02^{a}	0.22 ± 0.02^{a}
B	0.36 ± 0.02^{a}	0.14 ± 0.01^{b}	0.17 ± 0.02^{b}	0.07 ± 0.01^{b}	0.21 ± 0.01^{a}
C	0.37 ± 0.02^{a}	0.12 ± 0.01^{b}	0.16 ± 0.01^{b}	0.06 ± 0.01^{b}	0.19 ± 0.01^{a}

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

Discussion

The evaluation of haematological and biochemical characteristics in fish has become an important means of understanding normal, pathological processes and toxicological impacts [3]. Haematological alterations are one of the first detectable and quantifiable responses to environmental change [20]. Haematological and biochemical profiles of blood can provide important information about the internal environment of the organism [21]. In this work a significant decrease was observed in the MCV values, decreasing from the control to the third group. The high WBC count recorded could be due to attempt by the fishes to fight against the pollutants and this led to the production of more antibodies (WBC) to improve the health status

of the organism. This agrees with [22] that the increase in WBC during acute and sub-lethal treatment may be due to stimulated lymphomyeloid tissue as a defence mechanism of the fish to tolerate the toxicity. The mean cell volume (MCV) of fish in Group C showed a decreased trend in values in comparison with the control. MCHC is an indicator of RBC swelling and the lowered MCHC during treatment might have resulted from release of young erythrocytes containing less haemoglobin into circulation.

Oxidative damage has been suggested to occur as a consequence of reactive oxygen species (ROS). A number of studies suggested that ROS can affect critical events associated with many disorders [23-25]. It gets special attention due to many factors such as drought, cold, heat, herbicides and heavy metals, because they harm the cell by raising the oxidative level through loss of cellular structure and function, hence demands the detoxification agents like enzymes such as; superoxide dismutase (SOD), catalase (CAT) and peroxidase and non-enzymatic antioxidants such as flavones, anthocyanin, carotenoids and ascorbic acid [26]. The formation of ROS is prevented by an antioxidant system: low molecular mass antioxidants (ascorbic acid, glutathione, and tocopherols), enzymes regenerating the reduced forms of antioxidants, and ROS-interacting enzymes such as SOD, peroxidases and catalases [27-28]. The SOD enzyme destroys the superoxide radical; however, as a result of that it creates hydrogen peroxide, which also has high toxic properties [29]. 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 [30].

Glutathione (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 non-protein thiol in living organisms, which plays a central role in coordinating the body's antioxidant defense processes. Excessive peroxidation causes increased glutathione consumption [31]. Reduced thiols have long been reported to be essential for recycling of antioxidants like vitamin E and vitamin C. The observed significant reduction in the GSH level of the liver of test fish relative to control revealed the likelihood of biodiesel to induce oxidative stress in the tissue. 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. These conjugates reveal toxic properties [32]. In this study, biodiesel quickly depletes hepatocyte glutathione levels, therefore, a potential agent to inhibit many enzymes, which lead to further lipid peroxidation.

SOD selectively eliminates superoxide radicals in dismutation reaction in which hydrogen peroxide is generated. Reduction of SOD activity also may be due to an inhibited biosynthesis of enzyme molecules by biodiesel or its metabolites and/or to the effect of hydrogen peroxide, which may directly alter its activity. 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 [33]. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS). Likewise, 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 each second [34]. The change in levels of CAT in various groups of tissues may be as a result of the presence of biodiesel. The observed changes in CAT activity were concentration dependent, decreasing with increasing concentration of biodiesel. It could be

viewed that the biodiesel, like fuel diesel, is capable of generating ROS which may predispose to oxidative stress.

Malondialdehyde (MDA) is generated from reactive oxygen species (ROS), and as such is assayed in vivo as a bio-marker of oxidative stress [35]. Malondialdehyde reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts, malondialdehyde is reactive and potentially mutagenic. It has been found in heated edible oils such as sunflower and palm oils. The significant increase in the levels of MDA lend credence to the view that biodiesel caused a reduction in the total antioxidant status by reactive oxygen species.

Conclusion

In conclusion, toxicological effect of biodiesel on haematological properties is limited. However, the role of biodiesel in the reduction of antioxidant status is indicative of oxidative stress caused by reactive oxygen species. It is my view that adequate precautions must be observed by biodiesel production plants to avoid spillage of biodiesel into water bodies.

References

1. Qian J, Wang F, Liu S, Yun, Z. In situ alkaline transesterification of cottonseed oil for production of biodiesel and nontoxic cottonseed meal. Bioresource Technology, 2008; 99(18), 9009-9012.
2. Wilson RW, Taylor EW. The physiological responses of freshwater rainbow trout, Onchorynchusmykiss, during acute exposure. J. Comp. Physiol. 1993; 163b: 38- 47
3. Sudova E, Piackova V, Kroupova H, Pijacek M, Svobodova Z. The effect of praziquantel applied per as on selected haematological and biochemical indices in Common carp (Cyprinuscarpio L.). Fish Physiology and Biochemistry. 2008; 35(4):599-605.
4. Hakiman M, Maziah M. Non enzymatic and enzymatic antioxidant activities in aqueous extract of different *Ficus deltoidea* accessions. J. Med. Plants Res. 2009; 3 suppl 3: 120-131.
5. Stankiewicz A, Skrzydlewska E, Sulkowska M, Sulkowski S, Effect of amifostine on lung oxidative stress after cyclophosphamide therapy, Bull. Vet. Pulawy, 2002; 46, 87
6. Huang MC, Chen CH, Peng FC, Tang SH, Chen CC. Alterations in oxidative stress status during early alcohol withdrawal in alcoholic patients. J Formos Med Assoc 2009; 108: 560–569.
7. Jain S, Mythily S, Ahmed RS, Arora VK, Banerjee BD. Induction of oxidative stress and histopathological changes by sub-chronic doses of triazophos. Indian J Biochem Biophys 2010; 47: 388–392
8. Ferrari CKB. Free radicals, lipid peroxidation and antioxidants in apoptosis: implications in cancer, cardiovascular and neurological diseases. Biologia 2000; 55: 581–590
9. Alamu OJ, Akintola TA, Enweremadu CC, Adeleke AE. Characterization of palm-kernel oil biodiesel produced through NaOH-catalysed transesterification process. Sci Res and Essay 2008; 3 (7) 308-311
10. Mitruka, BM, Rawnsley M. Materials and methods in hematology and clinical biochemistry. In: Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals. Masson Publishing Inc. 1977; USA. Pp 41-58.
11. Muthaya NM. Blood In: Human Physiology, 3rd Edition. Jaypee Brothers Medical Publishers (P) Ltd New Delhi, India. 2002; Pp 51 – 91.

12. Tiez NW. In: Clinical guide to laboratory tests, 2nd ed. Philadelphia, USA: W.B. Saunders Company; 1990; P. 554.
13. Gornal AG, Bardawill JC, David MM. Determination of serum proteins by means of biuret reaction J. Biol. Chem. 1949; 177: 751-760
14. Bird RP, Drapper HH, Valli VE. Toxicological evaluation of Malondialdehyde: a 12-month study of mice, J. Toxicol. Environ. Health 1982; 10: 897-905.
15. Jollow DJ, Mitchell JR, Gillette JR. Bromobenzene induced liver necrosis: Protective role of glutathione and evidence for 3,4-Bromobenzene oxide as the hepatotoxic metabolite. Pharmacology. 1974; 11: 151-169.
16. Sinha, KA Colorimetric assay of catalase. Anal. Biochem. 1971; 47: 389-394.
17. Misra HP, Fridovich I. The role of superoxide anion in the antioxidation of epinephrine and a simple assay of superoxide dismutase. J. Bio1. Chem. 1972; 24 17-3170
18. Steel RGO, Torrie, JH. Principles and procedures of statistics, McGraw Hill Book Company Inc. London 1960; *p*. 15.
19. Duncan DB. Multiple range and multiple F test Bionet. **11:**1-10
20. Joshi PK, Bose, M, Harish D. 2002. Changes in certain haematological parameters in a siluroid catfish Clarias batrachusLinn exposed to cadmium chloride. Pollution Resources 1955; 21 (2) 129-131.
21. Okomoda J, Ayuba VO, Omeji S. Heamatological Changes of *Clariasgariepinus*(Burchell, 1822) Fingerlings Exposed To Acute Toxicity of Formalin. *PAT,* 2010; *6 (1):92-101*
22. Ates B, Orun I, Talas ZS, Durmaz G, Yilmaz I. Effects of sodium selenite on some biochemical and haematological parameters of rainbow trout (*Oncorhynchusmykiss*, Walbaun, 1792) exposed to Pb^{2+} and Cu^{2+}. Fish Physiol. Biochem. 2008; 34(5):3-9.
23. Sies H Oxidative Stress: Introductory Remarks. In: Oxidative Stress. Academic Press, London. 1985.
24. Meister A. Glutathione metabolism and its selective modification. J Biol Chem 1988; 263: 17205-17208.
25. Zheleva A, Tolekova A, Zhelev M, Uzunova V, Platikanova M. Free radical reactions might contribute to severe alpha amanitin hepatotoxicity- A hypothesis. Med hypotheses 2007; 69: 361-367
26. Ashok KBS, Lakshman K, Jayaveera KN, Sheshadri Shekar D, Nandeesh R, Velmurugan C. Chemoprotective and antioxidant activities of methanolic extract of amaranthus spinosus leaves on paracetamol induced-liver damage in rats. Acta Med Sal 2010; 39: 68–74.
27. Baysal Z, Cenqiz M, Ozqonul A, Cakir M, Celik H, Kocyiqit A. Oxidative status and DNA damage in operating room personnel. Clin Biochem 2009; 42: 189–193.
28. Bennet C, Bettaiya R, Rajanna S, Baker L, Yallapragada PR, Brice JJ, White SL, Bokara KK. Region specific increase in the antioxidant enzymes and lipid peroxidation products in the brain of rats exposed to lead. Free Rad Res 2007; 41: 267–273.
29. Baker K, Marcus CB, Huffman K, Kruk H, Malfroy B, Doctrow SR, Synthetic combined superoxide dismutase:catalase mimetics are protective as a delayed treatment in a rat stroke model: a key role for reactive oxygen species in ischemic brain injury. J. Pharmacol. Exp. Ther. 1998; 284, 215 – 221.
30. Banci L, Benedetto M, Bertini I, Del Conte R, Piccioli M, Viezzoli MS. Solution structure of reduced monomeric Q133M2 copper, zinc superoxide dismutase (SOD). Why is SOD a dimeric enzyme? Biochemistry 1998; 37, 11780 – 11791

31. Cederbaum AI, Lu Y, Wu D. Role of oxidative stress in alcohol-induced liver injury. Arch Toxicol 2009; 83: 519–548.
32. Flora SJS. Structural chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure. Oxid Med Cel Longev 2009; 2(4): 191–206.
33. Franzoni F, Quiñones-Galvan A, Regoli F, Ferranini E, Galetta F. A comparative study of the in vitro antioxidant activity of statins. Int J Cardiol 2003; 90: 317–321.
34. Garrabou G, Inoriza JM, Morén C, Oliu G, Miró Ò, Martí MJ, Cardellach F. Mitochondrial injury in human acute carbon monoxide poisoning: the effect of oxygen treatment. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 2011; 29: 32–51.
35. Day BJ, Batinic-Haberle I, Crapo JD. Metalloporphyrins are potent inhibitors of lipid peroxidation. Free Radic. Biol. Med. 1999; 26, 730 – 736