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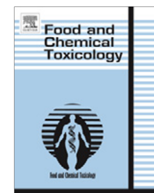

# Prophylactic effect of α-linolenic acid and α-eleostearic acid against MeHg induced oxidative stress, DNA damage and structural changes in RBC membrane

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ABSTRACT

The present study was undertaken to evaluate under *in vivo* condition the effects of α-linolenic acid and α-eleostearic acid against methyl mercury (MeHg) induced oxidative stress. Male albino rats were divided into six groups. Group 1 was under normal control and Group 2 was treated with methyl mercury chloride (MeHgCl; 5 mg/kg BW/day). Groups 3, 4, 5 and 6 were orally treated with different doses of the two fatty acids (0.5% and 1.0% of total lipid given for each kind of linolenic acid isomer) along with MeHgCl (5 mg/kg BW). Comet assay of blood lymphocytes showed that administration of α-linolenic acid reduced DNA damage significantly ($P < 0.05$). Results also showed that activity of antioxidant enzymes of plasma and brain tissue and total antioxidant capacity in plasma decreased significantly due to oxidative stress generated by MeHgCl. Administration in higher dose of both kind of linolenic acid restored all the activities of the antioxidant enzymes and also reduced lipid peroxidation and increased total antioxidant capacity in plasma. Both kinds of linolenic acid successfully maintained the RBC membrane integrity which was totally disrupted and became flat due to MeHgCl stress. α-Linolenic acid was more efficient antioxidant than α-eleostearic acid against oxidative DNA damage.

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## 1. Introduction

A rise in the industrial activity has gradually redistributed many toxic metals from the earth's crust to the environment, increasing human exposure to the toxic metals. Among them mercury (Hg); especially its organic form, methylmercury (MeHg), is the most malignant. MeHg exposure has been associated with oxidative stress *in vivo* (Ascher and Syversen, 2005; Crespo-López et al., 2007; Franco et al., 2007), in which mercury induces the generation of reactive oxygen species (ROS) (Sarafian, 1999) and alters the antioxidant defense system of cells (Kobal et al., 2007) by inhibiting their sulphydryl groups [-SH] or selenol groups [-SeH] (Mori et al., 2007; Farina et al., 2011). MeHg may also induce DNA damage by produce ROS (Jin et al., 2008) and/or inhibiting the DNA repair system (Cebulska-Wasilewska et al., 2005). It is well recognized that the oxidative stress is directly or indirectly associated with various diseases and aging (Liu and Mori, 1999). The brain is one of the most sensitive tissues to the oxidative stress because of its high contents of polyunsaturated fatty acid and neurotransmitters. Thus the oxidative stress induced neuronal damage and cell death plays critical role in pathogenesis of neurodegenerative disorders such as Alzheimer (Nunomura et al., 2006; Kidd, 2008) and Parkinson (Ebadi and Sharma, 2006; Danielson and Andersen, 2008) diseases. Again serum contains many different antioxidants that are important to general health and serum TAC has been considered as a suitable parameter for evaluating overall antioxidant status resulting from antioxidant intake, as well as production and consumption in response to oxidative stress (Nemec et al., 2000). In a previous study, antioxidant status in human plasma correlated negatively with the plasma concentration of Hg which in turn was positively correlated to the number of amalgam fillings in human subjects (Pizzichini et al., 2001). A number of studies established that oils containing polyunsaturated fatty acids (PUFA) play an active role in reducing oxidative stress (Watkins et al., 2007; Hassan et al., 2010; Dhar et al., 1999, 2007); as in case of PUFA, lipid peroxidation can be commenced by ROS production and other mechanisms that results in abstraction of an electron from PUFA.

*Abbreviations:* Hg, mercury; MeHg, methylmercury; MeHgCl, methylmercury chloride; CAT, catalase; PUFA, polyunsaturated fatty acid; ALA, α-linolenic acid; AEA, α-eleostearic acid; CLA, conjugated linoleic acid; EPA, ecosapentaenoic acid; DHA, docosahexaenoic acid; GSH, reduced glutathione; GPx, glutathione peroxidase; MDA, malondialdehyde; RBC, red blood cell; ROS, reactive oxygen species; SH-groups, sulphydryl groups; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; TCA, trichloro acetic acid; DNA, deoxyribo nucleuc acid; TAC, total antioxidant capacity; -SeH, selenol; -SH, thiol; FRAP, Ferric Reducing Ability of Plasma; AFM, atomic florescence microscope; EGM, erythrocyte ghost membrane.

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Subsequently, free radicals are constantly being generated and antioxidant defense mechanism neutralizes them, making them ineffective. α-Linolenic acid (9*c*,12*c*,15*c* 18:3) and α-eleostearic acids (9*c*,11*t*,13*t* 18:3), two typical tri-enoic fatty acid (two isomers of linolenic acid), are found in several seed oils such as linseed (*Linum usitatissimum*) oil (rich in 30–55% α-linolenic acid) and karela (*Momordica charantia*) seed oil (rich in 30–55% α-eleostearic acid), respectively and they also possess some important physiological and biochemical properties such as antioxidant, anti-carcinogenic and anti-inflammatory (Hassan et al., 2010; Dhar et al., 1999, 2007). In a recent study, in the author's laboratory, it was found that α-linolenic acid and α-eleostearic acids showed an ameliorative effect on the antioxidant status of both liver and kidney tissues against MeHg chloride (MeHgCl) stress (Pal and Ghosh, 2012).

The present study aimed at examining the effect of α-linolenic and α-eleostearic acid against oxidative stress generated by MeHgCl in brain tissue and plasma, DNA damage and structural changes in RBC membrane. A stress had been induced in rat body by the ingestion of MeHgCl and brain, plasma and erythrocyte ghost membrane (EGM) were analyzed. The fatty acid isomers were used in different doses to identify the potential effective dose for MeHgCl–diet interactions and to reveal the prophylactic or enhancing effects of dietary lipids on MeHgCl toxicity.

## 2. Materials and methods

### 2.1. Chemicals

The standard methyl mercury (II) chloride (MeHgCl) was procured from Sigma–Aldrich Chemicals Co., St. Louis, USA. The dose of MeHgCl (5 mg/kg BW/day) was chosen on the basis of the earlier studies (Sakamoto et al., 2004; Martins et al., 2009). The doses of linolenic isomers (0.5% and 1% of total lipid given) were also selected on the basis of the earlier studies (Dhar et al., 1999).

All other chemicals used were of analytical grade and procured from Merck India Ltd., Mumbai, India.

### 2.2. Extraction and quantification of α-linolenic acid and α-eleostearic acid

Authentic Linseeds (*L. usitatissimum*) and karela (*M. charantia)* seeds were obtained from the local market of Kolkata, India. Seeds were crushed in a coffee grinder and the oil was extracted with petroleum ether (b.p. 40–60 °C) solvent. The extracts of the sample were filtered and then concentrated by rotary evaporator. Free fatty acids present in the oil were neutralized by miscella refining process by adding 10% NaOH solution (20% excess of the required amount) at 40 °C for 30 min as described in our previous paper (Pal and Ghosh, 2012). The soap formed was removed by centrifugation and the organic phase was washed with water. Deacidified oil was recovered by distillation under vacuum and dried. The refined oil was then bleached with tonsil earth (1% w/w) obtained from P.T. Sud-Chemic (Jakarta, Indonesia) and activated carbon (0.2% w/w), supplied by E Merck India Pvt. Ltd. (Bombay, India), at 60 °C under vacuum for 20 min. After the bleaching operation, the oil was recovered by vacuum filtration and stored at −20 °C under nitrogen for feeding experiment.

### 2.3. Analysis of fatty acid compositions

The fatty acid (FA) compositions of the different dietary oils were determined by gas liquid chromatography (GLC, Agilent Technologies India Pvt. Ltd., Mumbai) techniques after preparing methyl ester of fatty acids present in the triglyceride molecule. The fatty acid compositions of dietary lipids are shown in Table 1.

### 2.4. Animals, diets and treatments

Male albino rats of Charles Foster strain (selected for the authenticity of the strain), weighing 100–130 g, were caged singly and provided with balanced diet and water *ad libitum*. The diets composed of fat free casein, 18%; fat, 20% (sunflower oil); starch, 55%; salt-mixture 4% (composition of salt mixture No. 12 (in g): NaCl 292.5, $KH_2PO_4$ 816.6; $MgSO_4$ 120.3; $CaCO_3$ 800.8; $FeSO_4{\cdot}7H_2O$ 56.6; KI 1.66; $MnSO_4{\cdot}2H_2O$, 9.35; $ZnCl_2$ 0.5452; $CuSO_4{\cdot}5H_2O$, 0.9988, $CoCl_2{\cdot}6H_2O$ 0.0476) cellulose 3%; one multivitamin capsule (vitamin A I.P. 10,000 units, thiamine mononitrate I.P. 5 mg, vitamin B I.P. 5 mg, calcium pantothenate USP 5 mg, niacinamide I.P. 50 mg, ascorbic acid I.P. 400 units, cholecalciferol USP 15 units, menadione I.P. 9.1 mg, folic acid I.P. 1 mg, vitamin E USP 0.1 mg) per kg of diet. The diets were adequate in all nutrients. The rats were maintained at 12 h light/12 h dark conditions. The animal experiment was carried under the supervision of the Animal Ethical Committee of the Department of Chemical Technology, University of Calcutta. The animals were divided into six groups consisting of six animals in each group. The first group served as normal control (Group C), received only vehicles (sunflower oil and deionized water) once per day. Rats in the Group 2 (Group M) were treated with MeHgCl (5 mg/kg BW) dissolved in sunflower oil by oral gavages once per day and served as treated control. Rats in the Groups 3 (Group AL) and 4 (Group AH) were treated with α-linolenic acid (ALA) (0.5% and 1.0% of total lipid given respectively) mixed with MeHgCl (5 mg/kg BW) by oral gavage once per day. Rats in the groups 5 (Group CL) and 6 (Group CH) were treated with α-eleostearic acid (AEA) (0.5% and 1.0% of total lipid given respectively) mixed with MeHgCl (5 mg/kg BW) by oral gavages once per day.

The animals were sacrificed after 15 days under anesthesia and kept in overnight (18 h) fasting before sacrifice. Blood was collected from hepatic vein into heparinized tube, brain were immediately excised, blotted, weighed and stored frozen (−40 °C) for analysis.

### 2.5. Measurement of antioxidative enzymes activity

The whole brain tissue was homogenized in 0.1 M Tris buffer (pH 7.0) and the homogenate was centrifuged at 105,000 g for 1 h. Superoxide dismutase (SOD) activity was assayed in plasma and brain homogenates and erythrocyte lysate according to the method described in our previous paper Pal and Ghosh (2012) and expressed as units/mg of protein. Catalase (CAT) activity was assayed in plasma and brain homogenates according to the method of Yumoto et al. (2000) and expressed as mmol of hydrogen peroxide decomposed/min/mg of protein. Reduced glutathione (GSH) levels in homogenates and plasma were quantified by the method described in our previous paper Pal and Ghosh (2012) and expressed as μg/mg of protein. Glutathione peroxidase (GPx) activity was assayed using method described in our previous work (Pal and Ghosh, 2012) and expressed as units/mg of protein. Total antioxidant capacity (TAC) was measured by FRAP method and expressed as μM (Benzie and Strain, 1999).

### 2.6. Plasma peroxidation

Plasma peroxidation was measured by the assay of thiobarbituric acid-reactive substances (TBARS) according to the method described in Dhar et al., 1999. The amount of malondialdehyde (MDA) formed was calculated by taking the extinction coefficient of MDA to be $1.56 \times 105\ M^{-1}\ cm^{-1}$.

### 2.7. Tissue lipid extraction and peroxidation

For lipid peroxide measurement, approximately 1 g of tissue was placed into a glass centrifuge tube (70 mL) for 2 min in a solvent mixture consisting of 10 mL of chloroform and 20 mL of methanol, and homogenized on ice. Then, 10 mL of chloroform was added and homogenization continued for another 30 s. Finally, 10 mL of redistilled water was added and the mixture was homogenized for 30 s. The tubes were then centrifuged for 20 min at 4000×*g*, and the chloroform layer was separated in a separatory funnel. A 2-thiobarbituric acid (TBA) test in chloroform phase was performed according to the method described in our previous work (Pal and Ghosh, 2012). Chloroform phase (2.5 mL) from the above lipid extraction was pipetted into a 30-mL autoclavable glass culture tube (Kimax; Kimble/Kontes, Vineland, NJ) having a Teflon-lined screw cap; 4 mL of the TBA reagent was added and the tube was capped tightly. Safety-shielded tubes were heated for 30 min in a boiling water bath and cooled in tap water. After cooling, 3.5 mL 5% trichloro acetic acid (TCA) was added to each tube, mixed thoroughly, and the tubes were then centrifuged at 3000×g.

### 2.8. Preparation of erythrocyte membranes

All procedures were done at 0–5 °C (typically on ice) and all centrifugations were performed in a Sorvall SS-34 rotor at 15,000 rpm unless specified. Rat red blood cells and hemoglobin free ghosts were prepared as described by Rose and Oklander (1996), except that the haemolysis buffer was 5 mM∼ $Na_2PO_4$ (pH 8), 0.01 mM $MgSO_4$ and the membranes were suspended for 10 min in this buffer before each centrifugation for complete removal of hemoglobin.

### 2.9. Measurement of membrane peroxidation

Membrane peroxidation was measured in erythrocyte ghost membrane by measuring MDA/g of protein in erythrocyte membrane according to the standard method stated above.

### 2.10. Measurement of protein content

Protein content was measured by the method described in our previous work (Pal and Ghosh, 2012) using BSA as standard.

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