Influence of ω-6/ω-3 rich dietary oils on lipid profile and antioxidant enzymes in normal and stressed rats

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To evaluate the influence of ω-6/ω-3 poly unsaturated fatty acid (PUFA) containing oils on lipid profile and endogenous antioxidant enzymes in normal and stressed (immobilization) rats, 28 day old male Wistar rats were fed for 45 days with fat enriched special diet (10% fat) prepared with sunflower oil (SO) — ω-6 rich, mustard oil (MO) — ω-3 rich and groundnut oil — control respectively. SO treated normal rats have significantly reduced total cholesterol, high density lipoprotein – cholesterol (HDL-C) and catalase thereby significantly increased the atherogenic index (AI) and lipid peroxidation (LPO). However, treatment with MO increased superoxide dismutase; decreased LPO significantly. Under stress conditions AI and LPO were significantly high with SO and significantly less with MO. In addition, SO decreased HDL-C whereas MO decreased non-HDL-C significantly. Results suggest a protective role against AI and LPO in normal and stress conditions in MO. The quantity of ω-3 fatty acids in dietary oil may play a crucial role in the body against atherogenicity. The findings signify that not just PUFA, but type of PUFA present in dietary oil used is important.

Keywords: Atherogenic index, Lipid peroxidation, Mustard oil, PUFA, Sunflower oil

Cardiovascular diseases (CVD) are the leading causes of death worldwide, accounting for an estimated 14 million deaths in 1990 and projected to cause 25 million deaths in 20201. It is predicted that economically developing countries such as India will see the greatest increase in cardiovascular deaths over next few decades2-4. CVDs in India cause 3 million deaths/year, accounting for 25% of all mortality3. With the urbanization and westernization of the society, stress is increasing day by day. Psychological stress induces chronic inflammatory process due to an atherosclerotic lipid profile with oxidation of lipids6,7. This in turn plays a significant role in the development of atherosclerotic heart disease (AHD)8. According to World Health Organization (WHO) estimates, by 2010, Asian Indians will represent 60% of the world’s cardiac patients.9. Early epidemiological studies in the subcontinent revealed that Indians had a very low prevalence of AHD, and it was presumed that they possibly had a low susceptibility to these disorders10. Studies during the last few decades showed a considerable rise in prevalence of these conditions in immigrant Asian Indians and some subsets of urban population in India belonging to upper socio-economic groups11-13.

Dietary fats have a significant role in the development of dyslipidemias14 and play an important role in development of atherosclerosis by modulating serum cholesterol concentration15-18. During the past few decades in most countries including India, traditional cooking oils have been replaced by cholesterol lowering unsaturated fats; many of them have high ω-6 poly unsaturated fatty acids (PUFA) content19,20. PUFAs are highly susceptible to hydrogen abstraction and become a source of reactive oxygen free radicals21,23. Oxidation of low density lipoprotein (LDL) lipids is a risk factor for atherosclerosis and AHD24,25. Vascular endothelium is highly susceptible to oxidative injury21 and the present global epidemic of AHD may be the result of faulty nutrition of the society26.

Dyslipidemia has been associated with high fat diet with high ω-6/ω-3 ratio of dietary fats26 and the consumption of these oils generates more oxygen radicals and thus excess intake of them is detrimental to health27,28. Excess use of PUFA- rich oils like sunflower or safflower oils, rich in linoleic acid (18:2ω-6) is harmful unlike what is misleadingly projected in the advertising media and the use of these oils as the sole cooking medium as is often prescribed...
by cardiologists, would give only a false sense of protection and would actually further aggravate the problem.\textsuperscript{29-37} Whereas mustard oil, one of the traditional cooking oil in India, is rich in \(\alpha\)-linolenic acid (18:3\(\omega\)-3), which has been reported to reduce the risk of AHD.\textsuperscript{3}

There are many unanswered questions and highly contradictory reports on dietary vegetable oils of our diet. Some are said to increase the heart risk while others claim to reduce it.\textsuperscript{29-37} Well-controlled studies using animal models are required to investigate the contradictory reports on dietary vegetable oils about their dyslipidemic and antioxidant property. Hence the present study is undertaken to evaluate the effect of \(\omega\)-6 rich [sunflower oil \(\omega\)- 6/\(\omega\)-3 (120:1)] and \(\omega\)-3 rich [Mustard oil \(\omega\)- 6/\(\omega\)-3 (1:2.1)] dietary oils on lipid profile and endogenous antioxidant enzymes under normal and stress conditions in rats.

**Materials and Methods**

*Experimental animals* — In-house laboratory bred 4 week old male Wistar rats (45±3g) were used. Animals were housed in polypropylene cage on clean paddy husk bedding and maintained under controlled temperature (20\(^\circ\)±2\(^\circ\)C) with an alternating 12 hr light: dark cycle (light on 06.00-18.00 hrs). Diet and water was provided *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC). Animal ethical guidelines and good laboratory practice guidelines were followed throughout the experimental period. In addition, all the precautions were taken to minimize pain and discomfort to the animals.

*Test oils* — Groundnut oil (GNO) – Safal; Sunflower oil (SO) - Gemini; and Mustard oil (MO) - Dhara were purchased locally.

*Test diets* — Diet 1: GNO (control), Diet 2: SO (\(\omega\)-6 rich) and Diet 3: MO (\(\omega\)-3 rich) (Table 1). The diets were prepared according to modified American Institute of Nutrition formulae (AIN-76).\textsuperscript{39} The antioxidant content of the experimental diets was same. The dose of the dietary vegetable oil was 10%. Diets were stored in a refrigerator and were prepared freshly every week. Individual group (n=6) were fed with respective diet for 45 days. Animals were provided with fresh diet daily and left over food was discarded.

*Experimental conditions* — Normal (N) group: Rats were maintained under standard laboratory conditions and fed with respective diet till the completion of the experiment. Stress (S) group: Rats were subjected to forced immobilization stress using rat restrainer for 7 hr/day. Under these conditions rats were fed with respective diet, till the completion of the experiment.

*Biochemical analysis* — The rats were allowed to feed on fat enriched diets *ad libitum*. The test diets with normal and test conditions were tested for their effects on lipid profile and antioxidant enzymes. At the end of 45 days, 2.0 ml of blood was withdrawn from the orbital sinus and the serum was separated from blood by centrifuging at 6000 rpm for 15 min. Lipid profile viz. total cholesterol (TC), high density lipoprotein (HDL-C) and triglyceride (TG) were estimated by biochemical kits from Ranbaxy using a semiautoanalyser. Non-HDL-C and atherogenic index (AI) was calculated according to the formula:

\[
\text{Non HDL-C} = \text{TC-HDL-C}^{40} \\
\text{AI} = \frac{\text{TC-HDL-C}}{\text{HDL-C}}^{41}
\]

*Activity of endogenous antioxidant enzymes* — After the withdrawal of blood, animals were sacrificed by cervical dislocation. Liver of the animals were perfused with normal saline and was dissected out, processed and 10% homogenates were prepared in saline (10%w/v), centrifuged and the supernatant was used for antioxidant enzyme assays.\textsuperscript{42}

*Total protein* — The protein contents of 10% liver homogenates were determined by using the Lowry’s method.\textsuperscript{43}

*Lipid peroxidation* (LPO) — Thiobarbituric acid reactive substances (TBARS) in homogenate were estimated by using standard protocol.\textsuperscript{44} Briefly, the homogenate was incubated with 15% trichloroacetic acid (TCA), 0.375% thiobarbituric acid (TBA) and 5N HCl. Reactive substances (TBARS) in homogenate were estimated by using standard protocol.\textsuperscript{44} Briefly, the homogenate was incubated with 15% trichloroacetic acid (TCA), 0.375% thiobarbituric acid (TBA) and 5N HCl. The rate of formation of thiobarbituric acid reactive substances (TBARS) and hydroxy radical (-OH) generated was determined using the Lowry’s method.

<table>
<thead>
<tr>
<th>Diet</th>
<th>GNO diet</th>
<th>SO diet</th>
<th>MO diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>12.5</td>
<td>12.5</td>
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</tr>
<tr>
<td>Sucrose</td>
<td>47.5</td>
<td>47.5</td>
<td>47.5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Groundnut oil</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Mustard oil</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>AIN mineral mix</td>
<td>3.5</td>
<td>3.5</td>
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<tr>
<td>AIN vitamin mix</td>
<td>1</td>
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<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
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</tr>
</tbody>
</table>

\(^{a}g/100g\) weight

GNO — Groundnut oil, SO — Sunflower oil, MO — Mustard oil

Table 1 — Diet composition.\textsuperscript{39}
hydrochloric acid (HCL) at 95°C for 15 min; the mixture was cooled, centrifuged and the absorbance of the supernatant measured at 532 nm against appropriate blank. The amount of LPO was expressed as nmoles/mg of protein. The amount of LPO was determined by using ε = 1.56 × 10^5 M^−1 cm^−1 and expressed as nM of TBARS/mg of protein^45^.

**Catalase** — The catalase activity was determined spectrophotometrically according to the standard protocol^46^ Briefly, to 1.95 ml of 10 mM hydrogen peroxide (H_2O_2) in 60 mM phosphate buffer (pH = 7.0), 0.05 ml of the liver homogenate was added and degradation of H_2O_2 was followed at 240 nm/min and the rate of decomposition of H_2O_2 was calculated using the formula k=2.303/∆t x log (A_1/A_2) s^−1 followed by calculation of catalase in terms of units/mg of protein. A unit of catalase is defined as the quantity, which decomposes 1.0 µmole of H_2O_2/min (pH=7.0) at 25°C, while this H_2O_2 concentration falls from 10.3 to 9.2 mM.

**Superoxide dismutase (SOD)** — SOD activity was determined based on the ability of SOD to inhibit the auto-oxidation of epinephrine to adrenochrome at alkaline pH^47^ Briefly, 25 µl of the supernatant obtained from the centrifuged liver homogenate was added to a mixture of 0.1 mM adrenaline in carbonate buffer (pH=10.2) in a total volume of 1 ml and the formation of adrenochrome was measured at 295 nm. The SOD activity (U/mg of protein) was calculated by using the standard plot.

**Statistical analysis** — Results were expressed as mean ± S.E. Statistical analysis involving three groups was performed by means of analysis of variance (ANOVA) followed by Bonferroni test. P value at < 0.05 was considered as statistically significant. All the data were processed with graph pad prism version 5.00 software.

**Results**

**Serum lipid profile** — Compared to normal control, normal rats fed diets containing SO diet had significantly lowered levels of TC, HDL-C; increased TC:HDL-C and AI (Table 2). However, MO diet non-significantly increased HDL-C, decreased TG, non-HDL-C, TC:HDL-C and AI (Table 2). Whereas compared to stress control, stressed rats fed SO diet have not shown significant decrease in TC but have shown significant decrease in HDL-C; increase in TC:HDL-C and AI (Table 2). On the other hand, stressed rats fed MO diet produced non-significant decrease in TC, TG; significant decrease in non-HDL-C, TC:HDL-C and AI (Table 2).

**Liver antioxidant enzymes** — Compared to normal control, normal rats fed diets containing SO diet had significantly lowered levels of catalase (Fig. 1b) and increased LPO (Fig. 1c). However, MO diet significantly increased SOD (Fig. 1a) and decreased LPO (Fig. 1c). Similarly, compared to stress control, stressed rats fed SO diet had significantly lowered levels of catalase (Fig. 1b) and increased LPO (Fig. 1c). However, MO diet had significantly increased SOD (Fig. 1a) and decreased LPO (Fig. 1c).

**Discussion**

Hypocholesterolaemic activity of SO diet (Table 2) observed in the present study may be due to the presence of linoleic acid content (ω-6 PUFA) which is known to be hypocholesterolaemic^16^ Feoli et al^48^, have also observed a decrease in serum cholesterol level in rats fed with ω-6 PUFA rich oil. Presently many people in India are consuming sunflower oil due to its hypocholesterolaemic property. But the decrease in HDL-C by SO diet played a major role in increasing AI (Table 2). Besides, SO diet under the stress conditions has not shown significant decrease in cholesterol and produced a further increase in AI as well (Table 2). Decrease in catalase (Fig. 1b) and increase in LPO (Fig. 1c) observed in the study by SO diet under normal and stress conditions, signifies the potential role of ω-6 PUFAs in free radical

<table>
<thead>
<tr>
<th>Parameters</th>
<th>GNO diet</th>
<th>SO diet</th>
<th>MO diet</th>
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<tbody>
<tr>
<td>TC (A)</td>
<td>62.72±1.5</td>
<td>55.75±1.6*</td>
<td>60.30±1.4</td>
</tr>
<tr>
<td>(B)</td>
<td>71.21±1.1</td>
<td>69.84±2.2</td>
<td>65.99±1.6</td>
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<tr>
<td>TG (A)</td>
<td>116.8±2.5</td>
<td>121.1±4.8</td>
<td>113.7±2.4</td>
</tr>
<tr>
<td>(B)</td>
<td>140.5±6.2</td>
<td>135.7±4.4</td>
<td>125.2±3.8</td>
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<tr>
<td>HDL-C (A)</td>
<td>22.63±0.7</td>
<td>18.0±4.6**</td>
<td>24.17±0.5</td>
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<tr>
<td>(B)</td>
<td>23.04±0.3</td>
<td>20.47±0.8*</td>
<td>24.08±0.5</td>
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<tr>
<td>Non HDL-C (A)</td>
<td>40.09±1.47</td>
<td>37.63±1.50</td>
<td>36.13±1.17</td>
</tr>
<tr>
<td>(B)</td>
<td>48.17±0.5</td>
<td>49.32±2.03</td>
<td>41.91±0.07*</td>
</tr>
<tr>
<td>TC:HDL-C (A)</td>
<td>2.78±0.09</td>
<td>3.09±0.11*</td>
<td>2.5±0.05</td>
</tr>
<tr>
<td>(B)</td>
<td>3.09±0.1</td>
<td>3.43±0.13*</td>
<td>2.74±0.01*</td>
</tr>
<tr>
<td>AI (A)</td>
<td>1.78±0.09</td>
<td>2.09±0.11*</td>
<td>1.54±0.05</td>
</tr>
<tr>
<td>(B)</td>
<td>2.09±0.1</td>
<td>2.43±0.13*</td>
<td>1.74±0.01*</td>
</tr>
</tbody>
</table>

Units: *mg/dl; †Ratio; ‡Units
P values: *<0.05, **<0.01 as compared with respective GNO diet (normal and stress)
One–way ANOVA followed by Bonferroni test.
Moreover, it has been reported that an increase in the dietary intake of ω-6 fatty acids shifts the physiological state to one that is prothrombotic, proaggregatory, proconstrictive, and proinflammatory state which may increase the risk to develop AHD. An imbalance of ω-6 and ω-3 PUFA in the peripheral blood causes overproduction of proinflammatory cytokines. Similarly, both ω-6 PUFA and stress have free radical generating nature. Therefore, the increased consumption of ω-6 PUFA under stressful conditions can increase the oxidative stress which is mainly due to the depletion of endogenous antioxidants. Excess free radical generation by ω-6 PUFA increases the susceptibility of LDL –C to oxidative modifications which could play an important role in the pathogenesis of several diseases.

ω-3 fatty acids have anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic and vasodilatory properties. Because of their ability to inhibit inflammatory pathways and suppress the expression of a large number of genes related to lipid metabolism, ω-3 fatty acids are being considered as therapeutic agents in dyslipidemia, the metabolic syndrome, type-2 diabetes etc. In the present study, there was non-significant hypocholesterolaemic activity in MO diet fed stressed rats (Table 2). Significant decrease in LPO (Fig. 1c) and increase in SOD (Fig. 1a) under normal condition and significant decrease in AI (Table 2), LPO (Fig. 1c) and increase in SOD (Fig. 1a) under stress condition signifies the beneficial effect of ω-3 fatty acids present in the MO diet and shows its importance in reducing the free radicals. As the oxidative modifications of LDL are crucial for the initiation of atherosclerosis, a significant reduction in non-HDL-C levels by MO diet fed stressed rats (Table 2) may provide prevention mechanisms against the CVD.

The present study highlights significance of an optimum ω-6/ω-3 ratio of fats in diet. These findings are in agreement with previous hospital based case control study report on dietary oils. SO diet though reduces cholesterol, possess increased AI and free radical generating property. Hence, consumption of high content of ω-6 PUFA may aggravate the occurrence of AHDs, this effect being worse when accompanied with stress. Present study also claims protective role of ω-3 rich MO diet against AI and LPO under normal and stress conditions. It can be concluded that the quantity of ω-3 fatty acids in the dietary oil must be playing a crucial role in the body against atherogenicity. The present results signify that it is not just PUFA, but the type of PUFA present in the dietary oil used which is important.

**Acknowledgement**

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**References**