Increased Unsaturated Fatty Acid Levels in Liver and Muscle of Guinea Pig Induced by Vitamin-E, ALA and Linalool

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Abstract: This study aimed to determine the protective effects of intraperitoneally administered vitamin E, lipoic acid and linalool on the levels of fatty acids in guinea pig liver and muscle under oxidative stress induced by hydrogen peroxide. While the level of total unsaturated fatty acid in the vitamin E, lipoic acid and linalool groups significantly increased (p < 0.01) as compared to the H₂O₂ group but its level slightly increased (p < 0.05) as compared with the control, H₂O₂ + vitamin E, H₂O₂ + lipoic acid and H₂O₂ + linalool groups in liver. The level of polyunsaturated fatty acid (PUFA) in H₂O₂ + vitamin E, H₂O₂ + lipoic acid and H₂O₂ + linalool groups increased in comparison with H₂O₂, decreased in comparison with vitamin E and lipoic acid groups in both tissues. In muscle, the level of unsaturated fatty acid in the H₂O₂ + vitamin E, H₂O₂ + lipoic acid and H₂O₂ + linalool groups increased in comparison with H₂O₂ group, but its level in the same groups decreased in comparison with vitamin E, lipoic acid and linalool groups. Vitamin E, lipoic acid and linalool could be used in therapeutic approaches, aimed at limiting the damages from oxidation reactions in unsaturated fatty acids.

Keywords: Vitamin E, lipoic acid, linalool, guinea pig, liver

Introduction
Reactive oxygen species (ROS) are known to play multiple roles in physiological and pathological states and are constantly produced in living organisms (Darr and Fridovich, 1994). The highly reactive superoxide radical and hydrogen peroxide may be toxic to cells by direct attack at the molecular level or indirectly by generating reactive species such as the hydroxyl radical (Brennessen et al., 1997). These radicals may cause oxidative damage virtually to any biomolecule (Kowaltowski and Veracini, 1999). Lipids, especially polyunsaturated fatty acids, are preferential targets for such oxidative damage (Douillet et al., 1998). If unsaturated fatty acids react with ROS in living cells, the resulting defects in membrane function may cause cell death (Gurr and Harwood, 1991). Despite the destructive potential of ROS, cells have developed defense mechanisms to prevent or limit oxidative injury. These mechanisms include several enzyme systems and antioxidants such as vitamin E and carotenoids, which prevent lipid peroxidation (Sobajic et al., 1998).

Vitamin E is present in the lipid bilayers of biological membranes and prevents oxidation of various compounds including unsaturated fatty acid (McCay, 1985). α-Lipoic acid is a naturally occurring free radical scavenger that has been shown to regenerate endogenous antioxidants, such as vitamin E and increase glutathione formation (Binnwenda, 1997). Recent reports indicate that lipoate exerts its therapeutic effect in pathological conditions involving free radicals (Kostov et al., 1999; Rudich et al., 1999).

Linalool is a monoterpene compound reported to be a major component of essential oils in various aromatic species. Several linalool producing species are used in traditional medicines. Among these is Uleianthus suaveolans G. Dom (Labiateae), which is used as an anticonvulsant in the Brazilian Amazon. Psychopharmacological evaluations of these compounds in vivo showed that they have marked dose-dependent sedative effects on the central nervous system, including hypnotic, anticonvulsant and hypothermic properties (Re et al., 2000). However, protective effect of linalool against the decrease of unsaturated fatty acid is not reported in the literature.

The objective of our work was to study the protective effects of intraperitoneally administrated vitamin E, lipoic acid and linalool on the levels of fatty acids in liver and muscle of guinea pig with oxidative stress induced by hydrogen peroxide.

Materials and Methods
Animals: Ninety seven guinea pigs were used during the experiment. At the start of the experiment, the guinea pigs weighed 460-640 g and were aged 11 months. The animals were divided into eight groups and kept at 20°C. They were fed ad libitum diet including the ingredients shown in Table 1 during the experiment.

<table>
<thead>
<tr>
<th>Table 1: Diet composition</th>
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<tr>
<td>Ingredients</td>
</tr>
<tr>
<td>Wheat</td>
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<tr>
<td>Corn</td>
</tr>
<tr>
<td>Barley</td>
</tr>
<tr>
<td>Wheat bran</td>
</tr>
<tr>
<td>Soybean</td>
</tr>
<tr>
<td>Fish flour</td>
</tr>
<tr>
<td>Meat-bone flour</td>
</tr>
<tr>
<td>Pteleed</td>
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<tr>
<td>Salt</td>
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<tr>
<td>Vitamin mineral mix²</td>
</tr>
</tbody>
</table>

*Vit. A, B₂, D₂, E, K₁, K₂, B₆, B₉, nicotinamide, folic acid, biotin, Mn, Fe, Zn, Cu, I, Co, Se, antioxidant and Ca.

The first group was the control (9 animals), the second was intraperitoneally administrated hydrogen peroxide (16 animals, 24 mg kg⁻¹), the third group received vitamin E (12 animals, 24 mg kg⁻¹), the fourth group hydrogen peroxide plus vitamin E (12 animals, 12 mg kg⁻¹ H₂O₂ + 24 mg kg⁻¹ vitamin E), the fifth group dl-α-lipoic acid (12 animals, 3 mg kg⁻¹), the sixth group H₂O₂ plus dl-α-lipoic acid (12 animals, 12 mg kg⁻¹ H₂O₂ + 3 mg kg⁻¹ lipoic acid), the seventh group linalool (12 animals, 120 mg kg⁻¹) and the eighth group received H₂O₂ plus linalool (12 animals, 12 mg kg⁻¹ H₂O₂ + 120 mg kg⁻¹ linalool) daily. This intraperitoneal administration was performed every day for 6 weeks.

Samples were prepared from animals at the end of the treatment period, after overnight fasting. Each experimental guinea pig was anaesthetized with ether and tissue samples were collected. These samples were kept at -25°C until lipid extraction and further analyses were performed.

Lipid extraction: Total lipids were extracted with chloroform-methanol (2:1, v/v) using the method of Folch et al. (1957). The tissue samples were homogenized and 3 g of them was mixed with chloroform-methanol (2:1, v/v). Non-lipid contaminants in the lipid extracts were removed with a 0.88 % KCl solution. The extracts were evaporated in a rotary evaporator and stored at -25°C.

Determination of total lipid: Total lipid was determined by the method of Frings et al. (1972). Twenty μl of each solution of
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extracted lipids was treated with 200 μl of concentrated H₂SO₄
and boiled for 10 min. After cooling, 10 ml phosphovanilin reagent
was added. The mixture was incuabated at 37°C for 16 min and
the absorbance of the samples was then read at 540 nm (CECLIN 5000
spectrophotometer: A high quality grade of olive oil (Sigma, St.
Louis, MO, USA) was used as a standard. The total lipid was
calculated by reference to the standard curve.

Fatty acid analysis: Fatty acids in the lipid extracts were converted
into methyl esters by means of 2% H₂SO₄ (v/v) in methanol
(Christie, 1980). The fatty acid methyl esters were extracted three
times with n-hexane, then separated and quantified using a gas
chromatograph equipped with a flame-ionization detector (Unicon
610 gas chromatograph) attached to a Unicam 4816 computing
recorder. Chromatography was performed with a capillary column
(26 m in length and 0.22 mm in diameter, B 10x70), using
hydrogen as the carrier gas (flow rate 0.6 ml min⁻¹). The
temperature of the column, detector and injection port was 195,
280 and 240 °C, respectively. Identification of the individual
methyl esters was performed by frequent comparison with
authentic standard mixtures analysed under the same conditions.

Statistical analysis: Data were reported as means ± SE. Statistical
analysis was performed using SPSS 8.0 Software. Analysis of
variance (ANOVA) and LSD test were used for comparison
between groups.

Results
At the end of the 6th week, the mean body mass of each
experimental group did not significantly differ. Mortality in control,
vitamin E, linool acid and groups after 6 weeks was nil but reached
25% in H₂O₂ and H₂O₂ + linool groups and 17% in linool,
H₂O₂ + vitamin E and H₂O₂ + lipic acid groups.

The effect of vitamin E, lipic acid and linool in the levels of
fatty acids in liver due to oxidative stress-induced by H₂O₂ are
reported so (Table 2). While the level of stearic (18:0) acids in the
H₂O₂ and H₂O₂ + lipic acid groups increased significantly (p < 0.05)
compared with the control, vitamin E, lipic acid and linool groups,
they increased slightly (p < 0.05) in comparison with
H₂O₂ + vitamin E and H₂O₂ + linool groups. The levels of total
saturated fatty acids did not differ between the control,
H₂O₂ + vitamin E, lipic acid, H₂O₂ + lipic acid and H₂O₂ + linool
groups, but were highest in the H₂O₂ group.

The total saturated fatty acid levels were reduced (p < 0.05) in the
vitamin E and linool groups compared to the H₂O₂ + vitamin E
and H₂O₂ + linool groups. The proportion of linoleic acid (18:2)
in the control, vitamin E, H₂O₂ + vitamin E, lipic acid, H₂O₂ + lipic
acid and linool groups increased slightly (p < 0.05) in comparison
with H₂O₂ + linool group, which increased significantly (p < 0.01)
in the same groups in comparison with the H₂O₂ group. In
addition, the level of arachidonic acid (20:4) in the vitamin E, lipic
acid, H₂O₂ + vitamin E and linool groups increased slightly
(p < 0.05) in comparison with the lipic acid, H₂O₂ + lipic acid and
H₂O₂ + linool groups, but increased significantly (p < 0.01) in comparison
with the control and H₂O₂ groups.

Docosahexaenoic acid (22:6) in the control and H₂O₂ groups
decrease in comparison with other groups. Total PUFA and
unsaturated fatty acid levels in the H₂O₂ group were minimal. On
the other hand, total unsaturated fatty acid levels did not differ
significantly between the control, H₂O₂ + vitamin E, H₂O₂ + lipic
acid and H₂O₂ + linool groups, but levels in the vitamin E, lipic
acid and linool groups increased slightly (p < 0.05) in comparison
with these same groups. Total PUFA levels did not differ between
the control, H₂O₂ + vitamin E, lipic acid, H₂O₂ + lipic acid, linool
H₂O₂ + lipic acid, and H₂O₂ + linool groups. Levels in the H₂O₂ + vitamin E
and H₂O₂ + linool groups were reduced in comparison with the
vitamin E and linool groups. The effects of vitamin E, lipic acid
and linool on the level of fatty acid in muscle tissue with
oxidative stress-induced H₂O₂ are reported (Table 3). Myristic acid
(14:0) was high in the H₂O₂ group. Its level did not differ
between the control, vitamin E, lipic acid and H₂O₂ + lipic acid
groups. While 18:0 levels in the control, H₂O₂ + lipic acid and
H₂O₂ + linool groups increased in comparison with the vitamin E,
H₂O₂ + vitamin E and lipic acid groups, they decreased slightly
(p < 0.05) in comparison with the H₂O₂ group. Levels of 18:0 did
not differ between the control, H₂O₂ + vitamin E and linool
groups, but increased in the lipic acid, H₂O₂ + lipic acid and
H₂O₂ + linool groups.

The proportion of 18:2 in the lipic acid and control groups
increased in comparison with the H₂O₂, H₂O₂ + lipic acid and
H₂O₂ + linool groups, but decreased in comparison with the
vitamin E, H₂O₂ + vitamin E and linool groups. The level of 20:4
was lovest in the H₂O₂ group. However, its level in the
H₂O₂ + vitamin E and H₂O₂ + linool groups were reduced as
compared with the vitamin E and linool groups. The level of 22:6
was also lovest in the H₂O₂ group. While the level of 22:6 in the
vitamin E and linool groups increased significantly (p < 0.01) in
comparison with the H₂O₂ + vitamin E, control and H₂O₂ + linool
groups. Total unsaturated fatty acid levels were highest in the H₂O₂
groups, but minimum in the vitamin E and linool groups.

The level of unsaturated fatty acid was minimum in the H₂O₂
group. Its level in the vitamin E and linool groups increased
slightly (p < 0.05) in comparison with the H₂O₂ + vitamin E and
H₂O₂ + linool groups, but increased significantly (p < 0.01) in
comparison with the control and H₂O₂ + lipic acid groups. The
level of PUFAs did not differ between the control, H₂O₂ + vitamin E,
lipic acid, linool and H₂O₂ + linool groups. However, its level in the
H₂O₂ + lipic acid and H₂O₂ + vitamin E groups was reduced in
comparison with the vitamin E and lipic acid groups.

Discussion
We have previously shown that the levels of saturated fatty
acids slightly decrease, but unsaturated fatty acids slightly
increase in rat and lamb tissues after dietary and
intraperitoneally-administered vitamin E, without any other
treatment (Yilmaz et al., 1997a; Yilmaz et al., 1997b; Celik et al.,
1999; Dilisiz et al., 1997). This effect of vitamin E on fatty acids
becomes clearer during hydrogen peroxide-induced oxidative
stress in this study. α-tocopherol is an antioxidant that prevents
biological membranes from undergoing oxidative damages, due
to its ability to quench lipid peroxides, thereby protecting cellular
structures from attack by free radicals (Traber and Sies, 1996).
Both isolated polyunsaturated fatty acids and those incorporated
into lipid are readily attacked by free radicals, becoming oxidized
into lipid peroxides. In contrast, both monounsaturated and
saturated fatty acids are more resistant to free radical attack;
indeed, it has been suggested that increased consumption of the
later instead of PUFA will render circulating lipoproteins less
sensitive to peroxidation (Reaven, 1991).

Hydrogen peroxide may be toxic to cells by direct attack at the
molecular level, or indirectly by generating secondary reactive
species such as hydroxyl radicals (Brennemann et al., 1997). The
significant reduction in PUFA levels in the hydrogen peroxide
treated groups observed in this study agrees with previous
findings. Chow et al. (1999) reported that vitamin E can directly
regulate hydrogen peroxide production in mitochondria and
suggested that the over-production of mitochondrial ROS is the
initial event leading to the tissue damage observed in Vitamin E
deficiency syndromes. Hydrogen peroxid may accumulate, leading
to a condition of mitochondrial oxidative stress (Kowaltowski et al.,
1999). In our study, vitamin E supplementation restored fatty acid
distribution close to that of the control group. These results clearly
demonstrate that unsaturated fatty acid peroxidation occurs in vivo in
H₂O₂-induced oxidative stress in the absence of lipidic acid
supplementation. At the same time, intraperitoneonal lipic acid
supplementation restores fatty acid distribution in H₂O₂ + lipic acid
groups close to that of the control
group. Magen et al. (1997) reported that feeding rats with the
linear aldehyde reduced malondialdehyde, an indicator of lipid
peroxidation. This is a free radical-induced process
Table 2: The effects of vitamin E, lipic acid and linoleal on the level of fatty acids in the liver tissues with oxidative stress induced by H$_2$O$_2$ (%).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Control</th>
<th>H$_2$O$_2$</th>
<th>Vitamin E</th>
<th>Lipic acid</th>
<th>Linoleal</th>
<th>Lin + H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td>1.21±0.36</td>
<td>2.12±0.12</td>
<td>3.79±0.17</td>
<td>4.04±0.11</td>
<td>1.22±0.13</td>
<td>1.22±0.13</td>
</tr>
<tr>
<td>Unsaturated</td>
<td>1.39±0.33</td>
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<td>1.39±0.33</td>
<td>1.39±0.33</td>
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</tr>
</tbody>
</table>

Table 3: The effects of vitamin E, lipic acid and linoleal on the level of fatty acids in the muscle tissues with oxidative stress induced by H$_2$O$_2$ (%).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Control</th>
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</tr>
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leading to oxidative deterioration of polysaturated lipids. Under normal physiological conditions, low concentrations of lipic peroxides are found in tissues. Free radicals react with lipids and cause peroxidative changes that result in enhanced lipid peroxidation (Grotti, 1988; Pyror, 1973). Thios are thought to play a pivotal role in protecting cells against lipid peroxidation (Haenen, 1989). Lipic acid effectively reduces the amount of hydroxyl radicals generated by the Fenton-type reaction and also scavenges peroxide and superoxide radicals (Sumathi et al., 1993). Our present observations show that lipic acid administration eventually results in a decrease in 18:2, 18:3, 20:4, 22:6, total unsaturated fatty acid and PUFA levels, thus substantiating the antioxidant properties of lipic acid. The results clearly demonstrate that unsaturated fatty acid peroxidation occurs in the tissues in H$_2$O$_2$-induced oxidative stress in the absence of lipic acid supplementation. The later inhibits the decrease in unsaturated fatty acids and restores fatty acid distribution close to that of the control group.

Although the dose of vitamin E used was approximately 8 times greater than that of lipic acid, the present study illustrates that lipic acid administration may be more effective than vitamin E in preventing lipid peroxidation in tissues. This may be attributed to the bioactivity of lipic acid directly reacting with various reactive oxygen species, as well as its ability to interfere with oxidation processes in lipid and the aqueous cellular compartment (Packer et al., 1995; Kagan et al., 1992). Lipic acid is a smaller molecule than vitamin E and is soluble in both water and fat. This is significant because water-soluble antioxidant nutrients (e.g. vitamin C) are found in the cell and fat-soluble antioxidants (e.g. vitamin E) are found on the cell membrane. Because lipic acid works both inside the cell and at the membrane level, it gives dual protection. Our results indicate that linoleal gives considerable protection against hydrogen peroxide induced oxidative stress in tissues. Linoleal is a monounsaturated fatty acid and is an essential component of cell membrane of various aromatic species. Since linoleal, which has a pleasant smell, is an effective antioxidant, it can be used as an additive in foodstuffs to protect against oxidative damage.

In conclusion, the results suggest that lipic acid and vitamin E could be used in therapeutic approaches for limiting the damages from oxidative reactions in unsaturated fatty acids and for complementing existing therapies in the treatment of oxidative damage. Linoleal was seen to have antioxidant properties similar to vitamin E and lipic acid. A dose-dependent studies will be necessary to determine whether linoleal could have beneficial effects in humans similar to those observed in animals.

Acknowledgements

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References

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