Effects of α-lipoic acid on DNA damage, protein oxidation, lipid peroxidation, and some biochemical parameters in sub-chronic thinner-addicted rats

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Abstract: The present study was carried out to determine the effects of sub-chronic thinner addiction on the oxidant–antioxidant balance, the relation between toxicity and oxidative stress, and a possible protective effect of α-lipoic acid against thinner toxication in rats. Sprague–Dawley rats were divided into 5 groups as follows: control (K), olive oil (Z), α-lipoic acid (L), thinner (T), and α-lipoic acid + thinner (LAT). Each group was composed of 15 rats, and the study lasted 8 weeks. After completing the animal studies malondialdehyde (MDA), reduced glutathione (GSH), methemoglobin (MetHb), toluol, and mononuclear leucocyte damage levels; protein oxidation, nitric oxide (NOx) metabolites, total antioxidant capacity (TAC), glucose, total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were determined from blood specimens of the rats. The data obtained from the study were statistically analyzed using SPSS, and both ANOVA and Duncan tests were performed. P < 0.05 was accepted as statistically significant. The results indicated that α-lipoic acid has a protective and balancing effect against complications derived from thinner inhalation in rats.

Key words: Thinner, oxidative stress, α-lipoic acid, thinner inhalation

Introduction

Exposure to chemicals and environmental agents causes an increase in the formation of free radicals in the cells and this leads to oxidative stress. Reactive oxygen types could interact easily with biological compounds such as DNA, RNA, lipids, and proteins. Thinner, first synthesized by Fischer in 1864, is an organic volatile solvent whose toxic effect is derived from the formation of reactive oxygen species (ROS). Thinner is used by industry, and is also abused by children and young people who are volatile substance addicts, as it is cheap and easily obtainable. Thinner abuse is not only an important health problem; it has become a social problem in some countries (1). The main components of thinner used in Turkish industry are: toluene (63%), acetone (13%), isobutyl acetate (10%), isobutanol (7.5%), and butyl glycol (6.5%) (2,3). It is well-recognized that solvent abuse can result in sudden death and also causes metabolic disturbances and pathological changes in various
tissues including the brain, lungs, liver, kidneys, testes, and surrenals (1,4). Mattia et al. (5) reported that thinner taken by inhalation shows its effects in the intestines, liver, kidneys, adrenal glands, and central nervous system. The excess production or diminished neutralization of ROS damages proteins, lipids, and/or nucleic acids. A number of reports state that long-term usage of volatile compounds/mixtures causes permanent damage to the liver and lungs (6,7). On the other hand, the damage to cells caused by organic solvents occurs through the formation of ROS (5). The harm caused by ROS is conventionally determined by measuring lipid peroxidation. Lipid peroxidation by ROS causes the deterioration of membrane functions, and, as a result of this dysfunction, fluidity of the membrane decreases, membrane-bound proteins may be inactivated, and both intercellular Ca^{2+} and Ca^{2+}-Mg^{2+}-ATPase activity levels increase (5,8). The increase in peroxidation and the decrease in antioxidant capacity are important complications caused by thinner (5,9,10).

Free radicals induce the cross linking between DNA and proteins, damage between deoxyribose and the phosphate skeleton, and specific modifications between the purine and pyrimidine bases (11,12). Oxidation of the deoxyribose skeleton causes the release of base and induces fracture of the DNA chain. Thus, oxidative base modifications cause the mutations (13,14).

Lipoic acid (LA) is one of the well-known antioxidants. It plays a crucial role in the Krebs cycle by activating the pyruvate dehydrogenase complex (PDH), which is responsible for the formation of acetyl-CoA (15). LA can function in both lipid and aqueous ambient and can overcome free radicals such as free oxygen, superoxide, peroxide, hydroxyl radicals, hypochlorite, and peroxynitrite by neutralizing them (16). Additionally, LA can form stable complexes with Mn^{2+}, Cu^{2+}, Zn^{2+}, and Pb^{2+} and is able to inactivate these heavy metals as well.

The aims of the study were to determine the effects of sub-chronic thinner addiction on the oxidant–antioxidant balance, the relation between toxicity and oxidative stress, and the possible protective effect of α-lipoic acid against thinner toxication in rats.

Materials and methods

Ethical committee approval

This study was approved by the Afyon Kocatepe University Ethical Committee for the Use of Experimental Animals (reference number: 3307/085).

Experimental groups and test protocols

The experiments were carried out on 3-month-old Sprague–Dawley male rats (n = 75), with average weights of 260–310 g. The animals were kept under 12 h dark/12 h light conditions with 3 rats per cage. Test subjects were maintained under the same conditions. During the study they were fed standard rat food and given water ad libitum. They were fed twice daily at 0900 and 1900. Before starting the thinner experiments, rats were subjected to pre-experiments in order to prevent possible toxication severity complications.

The experiments lasted 8 weeks, and test subjects were divided into 5 groups. Each group was composed of 15 rats [group 1: control group (K), group 2: sub-chronic thinner inhalation group (T), group 3: sub-chronic thinner inhalation + α-lipoic acid group (LAT), group 4: α-lipoic acid group (L), group 5: olive oil group (Z)].

Control group (K): comprised 15 healthy rats. They were fed standard rat food for 8 weeks, and their weights were recorded regularly during the experiments.

Sub-chronic thinner inhalation group (T): treated with 5 mL of thinner, embedded in cotton wool, in an air-proof isolated condition including NaOH tablets. This was repeated twice daily, and treatment was finalized when 50% of the rats’ standing reflex disappeared.

Sub-chronic thinner inhalation + α-lipoic acid group (LAT): treated as T group and additionally administrated 100 mg/kg/day α-lipoic acid dissolved in 2 mL of olive oil. The α-lipoic acid was introduced by gastric gavage.

α-lipoic acid group (L): administered 100 mg/kg/day α-lipoic acid dissolved in 2 mL of olive oil by gastric gavage.

Olive oil group (Z): treated with 2 mL of olive oil only, by gastric gavage.
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Blood sampling
After 8 weeks the rats were examined; they were given no food overnight and were weighed before euthanasia. For euthanasia, they were anesthetized using 10 mg/kg xylazine HCl and 50 mg/kg ketamine HCl. Then their ribcages were opened and an average of 6–9 mL blood was taken directly from their hearts by heparinized injector. Blood samples were kept at 4 °C until transfer to the lab. From each blood sample, 2 mL were separated for analysis of mononuclear leucocytes separation, MDA, MetHb, and GSH. The remaining blood samples were centrifuged at 3500 rpm for 10 min to separate the plasma. These specimens were kept in the deepfreeze (−30 °C) until analysis. Protein oxidation, total antioxidant capacity, NO x metabolites, glucose, triglycerides, HDL, LDL, AST, and ALT levels were measured using plasma specimens.

DNA damage determination by comet assay
The comet assay is used to determine DNA damage in the nuclei due to exposure to mutagenic and genotoxic compounds (17–23). It is based on the migration of DNA molecules/particles that have different electrical charges in alkali pH. The level of migration indicates the level of damage to the DNA. The Kocyigit et al. (17) method for preparation and running of DNA fragments was followed. Mononuclear leukocytes were separated for use in the comet assay. In this method heparinized blood samples were leaked into Histopaque 1077 in the test tubes; after formation of a tiny layer, test tubes were centrifuged at 2100 rpm for 30 min (25 °C). Next, the middle layer (containing mononuclear leukocytes) was transferred into 1 mL of salinated phosphate buffer (pH 7.4) and mixed. The resulting mixture was centrifuged at 1600 rpm for 10 min (25 °C). After discharging the supernatant, the pellet was diluted 106 in mm3 by salinated phosphate buffer (pH 7.4). Then leukocytes were mixed with 0.5% low-melting agarose in PBS at 37 °C. Subsequently, 80 μL of this mixture was layered onto a slide precoated with a thin layer of 1% normal-melting-point agarose (NMA), covered immediately with a cover slip, and stored for 5 min at 4 °C to allow the agarose to solidify. After removing the cover slips, the slides were immersed in freshly prepared cold (4 °C) lysing solution (2.5 M NaCl; 100 mM EDTA-Na₂; 1% Na-laurylsarcosine; 10 mM Tris-HCl, pH 10–10.5; and 1% Triton X-100 with 10% DMSO added just before use) for at least 1 h. The slides were then electrophoresed (25 V/300 mA, 25 min) after immersion in freshly prepared alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA-Na₂, pH > 13) at 4 °C for unwinding (40 min). All steps were carried out under minimal illumination. After electrophoresis the slides were neutralized (0.4 M Tris-HCl, pH 7.5) for 5 min. The dried microscope slides were stained with ethidium bromide (2 μg/mL in distilled water; 70 μL/slide), covered with cover slips, and analyzed using a fluorescence microscope.

The images of 100 randomly chosen nuclei were analyzed visually. Observations were made at a magnification of 200× using a fluorescent microscope (Olympus, Japan). Each image was classified according to the intensity of the fluorescence in the comet tail and given a value of 0, 1, 2, 3, or 4 [from undamaged (class 0) to maximally damaged (class 4)] so that the total slide score would range from 0 to 400 arbitrary units (AU) (17,19).

MDA determination
MDA is a peroxidation product of free radicals. Draper and Hadley’s (24) double boiling method was used for MDA determination. This method is based on the interaction of thiobarbituric acid (TBA) and MDA and their maximum absorbance at 532 nm, spectrophotometrically.

Determination of MetHb level
MetHb level determination was performed by spectrophotometer following the methods described by Harrison and Jollow (25). Blood samples (75 μL each) were taken and mixed with 5 mL of phosphate buffer. After hemolysis, blood solutions were divided into 4 equal volumes in separate test tubes. To the first 2 tubes, 1 drop of KCN (10%) was added; to the other 2 tubes, 1 drop of K3 Fe(CN) 6 was added. Then the tubes were vortexed, and absorbance was recorded at 635 nm. The levels were calculated by the following equation:

\[ \text{% methemoglobin} = \frac{A_1 - A_2}{A_3 - A_4} \times 100. \]
Determination of protein oxidation

Protein oxidation was measured using both Levine et al. (26) and Ceylan et al. (27). This method is based on the color of hydrozone compounds, which occur in the reactions of carbonyl groups of oxidized proteins and 2, 4-dinitrophenylhydrazine.

Total antioxidant capacity

Total antioxidant capacity was determined by antioxidant assay kit supplied by Cayman Chemical (catalogue number: 709001) on Multiscan spectrum (Thermo) culture plaque readers; results were given as mM.

Determination of reduced glutathione (GSH)

Blood samples were hemolyzed using distilled water, and proteins containing no –SH group were precipitated using a solution containing 1.67 g metaphosphoricacid,0.2g ethylenediaminetetraacetic acid (EDTA), and 30 g NaCl in 100 mL of water. GSH was measured using the absorbance (412 nm) of the color yellow occurring in the supernatant by reacting –SH groups with DTNP [5, 5’-dithiobis (2-nitrobenzoic acid)] (28).

Determination of NOx

NOx levels in the plasma samples were determined using the methods of Miranda et al. (29) and Bülbül (30); this was based on the vanadium chloride (III)- Griess reaction.

Determination of biochemical parameters

Glucose, total cholesterol, triglycerides, HDL-cholesterol, and LDL-cholesterol levels were determined spectrophotometrically (Shimadzu UV-1601), and conventional methods were by commercial kits. AST and ALT levels were determined by Cobas Integra 400 autoanalyzer (Roche). Toluol levels were determined at the Acıbadem Labmed Central Lab (İstanbul) by GC-MS.

Statistical analyses

The data obtained from the study were subjected to statistical analyses using SPSS 13.0. The average results were given as mean ± SD. First, normality tests were performed, and the differences were determined by ANOVA; the Duncan test was used as a post-test. P < 0.05, P < 0.01, and P < 0.001 were accepted as statistically significant, more significant, and most significant values, respectively. Additionally, a correlation test was performed to determine relationships among the parameters (31).

Results

Variation in biochemical parameters

Glucose, cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, ALT, AST, MetHb, mononuclear leukocyte DNA damage, protein oxidation, total antioxidant capacity, GSH, and NOx levels from the 8-week period and from all groups examined are shown in Table 1. Statistical comparison values are also indicated in Table 1.

As seen in Table 1, glucose levels of the Z, L, T, and LAT groups were significantly higher when compared to the control group (P < 0.05). Triglycerides increased very significantly only in the Z group (P < 0.01). LDL-cholesterol levels increased significantly in all groups compared to controls (P < 0.01). The LDL levels in the LAT group were significantly higher than in the L and T groups (P < 0.01). AST levels in the LAT and T groups were higher than in the Z and L groups (P < 0.05). On the other hand, ATS levels in the LAT group were significantly lower than in the T group (P < 0.05). These results suggest that LA has a protective effect on the liver. When the MDA levels were observed, they increased in the T group very significantly when compared to the control group (P < 0.001). In contrast, the level in the LAT group was significantly lower than in the T group (P < 0.01) and declined in the control group. Mononuclear leucocyes DNA damage levels were very high in both the T and LAT groups when compared to the control group (P < 0.001). However, this damage declined in the LAT group when compared to the T group (P < 0.001). Protein oxidation levels increased significantly in the T group, compared to the control, Z, L, and LAT groups (P < 0.01). Total antioxidant capacity levels were lower in both the LAT and T groups compared to the control group (P < 0.01). However, its level in the LAT group was significantly increased compared to the T group (P < 0.001). Protein oxidation levels increased significantly in the T group, compared to the control, Z, L, and LAT groups (P < 0.01). Total antioxidant capacity levels were lower in both the LAT and T groups compared to the control group (P < 0.01). However, its level in the LAT group was significantly increased compared to the T group (P < 0.01), and its level was closer to the control group level. GSH levels were significantly high in the L group compared to the control, LAT, and T groups (P < 0.01). However, its level in the LAT group was significantly higher in the T group when compared to the other groups (P < 0.001). When the LAT and T groups were compared, the
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MetHb level was very significantly low in the LAT group (P < 0.001).

When we look at the relationship between toluol levels and other parameters of the T group (Table 2) there is a positive correlation between toluol level and MetHb, mononuclear leucocytes DNA damage, and MDA levels. In contrast, this relation was negative in terms of GSH and total antioxidant capacity levels.

On the other hand, there was a positive relation between MDA and protein oxidation levels and a negative relation between protein oxidation, GSH, and antioxidant capacity.

**Discussion**

Rats inhaled thinner twice daily without physical contact. During the inhalation rat activity slowed; they had difficulty walking, their inhalation was impaired, and they became aggressive. Apart from these effects, they began to lose a remarkable amount of weight after the 45th day of the experiments. Yamada et al. (32) reported a similar observation after the seventh day in their experiments.

Routine biochemical parameters showed that glucose levels increased in the Z, L, T, and LAT groups when compared to the control group, and its levels in the L and LAT groups were significantly higher when compared to the other groups (Table 1). These results suggest that thinner inhalation increased blood glucose levels even in those rats that were given LA. These findings are not compatible with the literature (33,34); however, detailed investigations should be carried out on this issue in order to increase the amount of information available.

LDL-cholesterol levels increased in all test groups when compared to control; however, HDL-cholesterol levels did not differ in all groups. Additionally, although plasma cholesterol level did not differ, the triglyceride level was highest in the Z group. Guzelian et al. (35) conducted research on 289 printing house workers who were exposed to less than 200 ppm of toluene for 8 h and reported that ALT and AST levels
increased. We also observed differences in these 2 parameters as observed by Guzelian et al. (35). As we know, these 2 parameters are important indicators of liver function. We observed that AST levels in the LAT and T groups increased significantly when compared to the Z, L, and control groups. On the other hand, AST levels in the LAT group significantly decreased when compared to the T group. These findings suggest that LA has a protective effect against thinner toxicity in the liver. As there was no difference in ALT levels in all groups these results should be studied in detail in order to clarify the harmless effect of thinner on this enzyme.

Ulakoğlu et al. (2,3) exposed rats to the thinner for 5 weeks, and they reported that MDA and 4-DHA levels increased significantly. Bozic et al. (36) exposed rats to toluene for 3, 7, and 11 days and looked at the red blood cells of the experimental rats. They reported that oxidative stress due to toluene induced the formation of MDA. In our study, MDA levels significantly increased in the T group when compared to the control group. However the level decreased in the LAT group and approached the control group level. Additionally, the positive relation between MDA and toluol levels may be due to the increase in lipid peroxidation resulting from an increase of toluol in the blood. The increment in lipid peroxidation in the T group is almost the same (37–39). In addition, the decrease in MDA levels in the LAT group related to the T group suggests that α-lipolic acid has both a protective effect and antioxidant capacity against harmful oxidation products.

Hemoprotein and oxyhemoglobin are significant sources of ROS in biological systems. This is due to electron transfer from their iron contents to oxygen (40). MetHb levels increased significantly in the T group when compared to the other test groups. However, MetHb level declined significantly in the LAT group in relation to the T group; there was no significant difference in the L and Z groups compared to the control group. As seen in Table 2, there is a positive relationship among MDA, toluol, and MetHb. This relationship may be due to inhaled toluol in the thinner.

The comet assay was chosen to determine DNA damage due to its low cost, rapidity, and sensitivity. Alfaro et al. (41) reported that DNA damage increased due to the thinner inhalation period. They also found a correlation among ascending DNA damage and MDA and declining GSH levels. Lipid

<p>| Table 2. Relationship with levels of blood toluol, DNA damage, and oxidant/antioxidant parameters. |
|-------------------------------------------------|----------------|----------------|-----------|--------|--------|--------|--------|</p>
<table>
<thead>
<tr>
<th>Toluol</th>
<th>MetHb</th>
<th>Mononuclear Leukocyte DNA damage</th>
<th>MDA</th>
<th>PO</th>
<th>GSH</th>
<th>TAK</th>
<th>NOx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluol</td>
<td>1</td>
<td>0.745</td>
<td>0.851</td>
<td>&lt;0.05</td>
<td>0.885</td>
<td>0.691</td>
<td>-0.869</td>
</tr>
<tr>
<td>MetHb</td>
<td>1</td>
<td>0.891</td>
<td>&lt;0.01</td>
<td>0.951</td>
<td>NS</td>
<td>-0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Mononuclear leukocyte DNA damage</td>
<td>1</td>
<td>0.715</td>
<td>0.640</td>
<td>-0.635</td>
<td>-0.917</td>
<td>0.120</td>
<td></td>
</tr>
<tr>
<td>MDA</td>
<td>1</td>
<td>0.779</td>
<td>-0.895</td>
<td>-0.851</td>
<td>0.634</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PO</td>
<td>1</td>
<td></td>
<td>-0.524</td>
<td>-0.734</td>
<td>0.453</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>1</td>
<td></td>
<td>0.694</td>
<td>-0.537</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAK</td>
<td>1</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOx</td>
<td>1</td>
<td></td>
<td>NS</td>
<td>0.303</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MetHb: methemoglobin; MDA: malondialdehyde; PO: protein oxidation; GSH: glutathione; TAC: total antioxidant capacity; NOx: nitric oxide.
hydroxyperoxides can directly induce DNA chain breaks. Additionally, lipid peroxyls and alchoxyl radicals cause base oxidation in DNA chains. The free radicals are generated during the MetHb formation in biological systems, and one of their potential targets is the DNA molecule. When oxygen-originated free radicals interact with DNA they cause base decrease or increase, chain breakage, frame shifts, and DNA–protein cross links. These abnormalities cause mutations, transcription or replication repressors, or permanent genetic variation (39). Our comet assay results indicated that DNA damage in the T and LAT groups was significantly high when compared to the control group. When the T and LAT groups were compared, however, the damage was significantly low in the LAT group. These results are compatible with the literature. As seen in Table 2 there is a positive correlation between increased oxidative stress and DNA damage. This may be due to thinner induction of oxidative stress. When we looked at the decrease in DNA damage in the LAT group we established that α-lipoic acid has a protective and/or suppressive effect on thinner toxicity.

Protein oxidation occurs with the covalent modifications and interactions between ROS or oxidative stress products and proteins. Interaction between thiol or amino groups in proteins and free radicals causes some modifications, and as a result of this event structural differentiation in proteins may occur through fragmentation and protein aggregations or cross linking in their structural shape (42). Our findings indicated that thinner inhalation by rats induced protein oxidation. The level was statistically significant in the T group when compared to the control group. However, we also observed that α-lipoic acid decreased protein oxidation. Our findings also indicated that there was a positive relation between MDA levels and protein oxidation (Table 2), and this relation may be due to oxidative chain reactions induced by toluol.

In fact, there are defending mechanisms called antioxidants present in serum, red blood cells, and different tissues. GSH plays very important protective and integrity roles as an antioxidant in the cells, tissues, and organs (43). Glutathione is present in 2 forms in the organism: reduced glutathione (GSH) and oxidized glutathione. Although it is present in all tissues, it is synthesized actively in the liver. Ulakoğlu et al. (2,3) reported that GSH levels in both lung and liver tissues of the thinner-exposed rats dropped.

LA is absorbed rapidly from the mouth and taken into cells. Free LA is reduced to dihydroliopioic acid (DHLA). DHLA is then reduced from cystine to cysteine, and the biosynthesis of GSH is accelerated. In this pathway, the indirect antioxidant effect of DHLA was observed (44,45), and the protective effect of α-lipoic acid against oxidative stress formed by Cyclosporine A was examined. When α-lipoic acid was administrated at 20 mg/kg/day for 21 days, GSH level increased steadily. In the present study total antioxidant capacity levels were lower in both the T and LAT groups when compared to the controls. However, total antioxidant capacity in the LAT group increased significantly compared to the T group. GSH levels were significantly high in the L group compared to the control, LAT, and T groups. These findings are compatible with the literature (45). As seen in Table 2 there is a negative relation among toluol levels and GSH and total antioxidant capacity. Additionally, a similar relationship was observed between MDA and protein oxidation and GSH and total antioxidant capacity. This may be due to increasing oxidative stress. Additionally, α-lipoic acid was unable to prevent a decrease in GSH over the 8-week period.

It was reported that NOx has a protective and regulator effect on lipid peroxidation, DNA damage, deterioration of enzymes functions, and lowering of oxidative damage (46,47). This study investigated NOx levels in the workers exposed to toluol, xylene, and methyl-ethyl ketone in a work environment (shoe and leather industries), and found that NOx levels were higher in workers than in control. Our findings showed that NOx metabolites in Z and T groups were high when compared to the controls; the levels were low in T and LAT groups compared to controls.

A number of pathways play a key role in thinner-induced oxidative stress. Alfaro et al. (48) demonstrated some of these pathways: metabolism of thinner components, enzymes (P450), metabolites (quinones), and inflammatory reactions. In addition, thinner works indirectly through a solvent-like action, which affects lipid membranes. These pathways are not exclusive of one another. Indeed,
all mechanisms contribute to the ability of thinner to induce oxidative stress.

In conclusion, thinner inhalation produces harmful effects in those who are exposed to it, and α-lipoic acid could be used as a supportive chemical in the classical treatment of oxidative stress. As seen in our study, α-lipoic acid has both a buffering and preventative effect on the possible complications of thinner toxicity. On the other hand, detailed investigations should be carried out on the issues that produced conflicting results the literature. It is hoped that our findings will help those who are exposed to thinner in their work and/or aid in the treatment of thinner addicts.

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