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The effect of lipoic acid on antioxidant status and lipid peroxidation in rats exposed to chronic restraint stress

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Abstract

This study was designed to investigate effect of alpha-lipoic acid (LA) on lipid peroxidation, nitric oxide production and antioxidants in rats exposed to chronic restraint stress. Twenty four male wistar rats, aged three months, equally divided into four groups: Control (C), the group treated with LA (L), the group exposed to restraint stress (S) and the group exposed to stress and treated with LA (LS). Restraint stress was applied for 21 days (1 h/day) and LA (100 mg/kg/day) was injected intraperitonally to the L and LS groups for the same period. Restraint stress significantly decreased brain copper/zinc superoxide dismutase (Cu,Zn-SOD) and brain and retina glutathione peroxidase (GSH-Px) and catalase (CAT) activities compared with the control group. Thiobarbituric acid reactive substances (TBARS), nitrite and nitrate levels were significantly increased in the tissues of the S group compared with the C group. LA produced a significant decrease in brain and retina TBARS, nitrite and nitrate levels of the L and LS groups comparing with their corresponding control groups. LA increased all enzyme activities in the tissues of the LS group respect with the S group. Our study indicated that LA is an ideal antioxidant candidate for the prevention of stress-induced lipid peroxidation.

Keywords: Restraint Stress, Lipoic Acid, Lipid Peroxidation, Antioxidant enzymes

INTRODUCTION

Stress is conceived as an aversive stimulus capable of altering physiological homeostasis, and the ability to cope with such stressful stimuli is a crucial determinant of health and disease (Masood *et al.* 2003). Intensive stress has detrimental effects on organism by causing cellular and tissue injury. The mechanisms underlying stress-induced tissue damages are not yet fully understood, however, accumulating evidence has implied that the production of free radicals plays a critical role in these processes (Liu *et al.* 1996, Liu and Mori 1999, Olivenza *et al.* 2000, Madrigal *et al.* 2002, Zaidi *et al.* 2003). Previous studies have indicated that stress stimulated numerous pathways leading to increased levels of free radicals (Liu *et al.* 1996, Liu and Mori 1999, Olivenza *et al.* 2000, Liu *et al.* 1994, Matsumoto *et al.* 1999). One of the reasons for the stress-induced enhancement of free

radicals may be the elevation of nitric oxide (NO) production (Matsumoto *et al.* 1999, McCann 1997). NO may interact with oxygen, superoxide anion, and thiol compounds, generating reactive nitrogen species (NO_x), peroxynitrite ($ONOO^-$) and s-nitrosothiols including s-nitrosoglutathione (GSNO). Lipid peroxidative effect of NO may be mediated through $ONOO^-$ which is a potent and long-lived oxidant (Chiueh and Rauhala 1999, Siu *et al.* 1999, Rubbo *et al.* 2000).

Under normal conditions, there is also a natural defense system provided by several enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) which performs a vital role for detoxification of free radicals. However, stress has been shown to cause depletion of the glutathione-based antioxidant defense and a decrease in the level of vitamin C (Zaidi *et al.* 2003, Zaidi and Banu 2004 Matsumoto *et al.* 1999). The deleterious effects of an imbalance between free radical production and the available antioxidant defence capacity, termed oxidative stress has been implicated in the stress as well as in pathogenesis of several disease states. One of the important consequences of oxidative stress is the peroxidation of membrane lipids. Since the retina and brain tissues include high content of polyunsaturated fatty acids, this reaction produce marked damage to the structure and function of cell membranes in these tissues (Matsumoto *et al.* 1999, Jain *et al.* 1991). Therefore, lipid peroxidation has supposed as the major biochemical alteration underlying oxidant-induced cell injury in stress including numerous diseases (Olivenza *et al.* 2000, Liu *et al.* 1994, Zaidi and Banu 2004, Shaheen *et al.* 1993).

The use of antioxidant rich food or antioxidant food supplements became immensely popular since many diseases have been associated with oxidative stress. Therefore, in the last decade, an increasing amount of attention has been focused on free radical scavengers that are able to protect against aberrant effects of free radicals. Thus, one of the natural molecules known to prevent or retard oxidation is a lipoic acid (LA) and, therefore, the lipoic acid/dihydrolipoic acid (LA/DHLA) redox couple has received considerable attention (Marangon *et al.* 1999). It is well-known that LA is readily absorbed from diet and also crosses the blood brain barrier (BBB) (Morikawa *et al.* 2001, Harnett *et al.* 2001).

So, recent studies have highlighted the potential of free LA and DHLA as powerful metabolic antioxidants that are able to scavenge the reactive oxygen species, to recycle other antioxidants (vitamin C, glutathione, and vitamin E). Additionally, LA functions as a cofactor in several mitochondrial multienzyme complexes involved in energy production (Marangon *et al.* 1999, Morikawa *et al.* 2001, Suzuki *et al.* 1991, Hagen *et al.* 1999). In the present study, the main goal was to investigate the beneficial effects of LA and whether this supplementation affected antioxidant status and oxidative stress. In this context, thiobarbituric acid reactive substances (TBARS) were measured as an indicator of lipid peroxidation. Additionally, Cu,Zn-SOD, GSH-Px and CAT activities were evaluated to determine the antioxidant defense system response to chronic restraint stress and to clarify the antioxidative potential of LA. Finally, nitrite/nitrate, stable nitric oxide metabolites, were also determined to evaluate the effect of LA on NO production.

MATERIALS AND METHODS

Preparation of animals: Twenty four healthy male albino rats, aged three months, were used in this study. They were equally divided into four groups: Control (C), the group treated with LA (L), the group exposed to restraint stress (S), the group exposed to restraint stress and treated with LA (LS). Animals were housed in a group of 6 rats in stainless steel cages at standard conditions (24 ± 2 ° C and 50 ± 5 % humidity) with 12 h light-dark cycle.

i. LA (Sigma, St. Louis, MO, USA; 100 mg/kg/day) was injected intraperitonally to the L and LS groups for 21 days.
ii. Physiologic saline was injected intraperitonally to the C and S group for 21 days.

Experimental Procedures: All experimental protocols conducted on rats were performed in accordance with the standards established by the Institutional Animal Care and Use Committee at Akdeniz University Medical School. The experiments were performed between 9:00-12:00.

Restraint stress: Restraint stress model was performed according to the methods described by Gamoro *et al.* (Gamoro *et al.* 1999). Rats were exposed to 1 hour of restraint stress daily for 21 days by placing the animals in a 25x7 cm plastic bottle. The bottle was fixed with plaster tape on the

outside so it was unable to move. There was 1.5 cm hole at one end of the bottle for breathing. Daily food and water consumption of every cage and weekly weight of individual rat were recorded during the feeding period. The mean daily food and water consumption was estimated from the recorded values.

Chemical analysis: At the end of the 21 days experimental period, rats were sacrificed by exsanguination via cardiac puncture**.** The isolated retina and brain tissues were placed in 2 ml and 4 ml of 50 mM potassium phosphate buffer, pH:7.4 at 0-4 °C. All tissues were rapidly sonicated in a thermally regulated sonicator (Branson Sonifier 250**,** G. Heinemann Ultraschall- und Labortechnik, Erwin-Rommel-Str. 42, D-73525 Schwäbisch Gmünd, Germany) for 1 min. A part of the tissue sonicates were centrifuged at 13.000 g for 10 min at 4 °C in an eppendorf microcentrifuge and the supernatants were used for the assay of TBARS nitrite, nitrate and protein. A part of the samples were sonicates at 700g for 10 min was used for the assay of CAT, remaining tissue sonicates were centrifuged at 20.000 g for 60 min at 0-4^{0}C and supernatants were employed for the enzymatic assay of GSH-Px and SOD.

TBARS Assay: TBARS levels were measured by a fluorometric method described by Wasowicz *et al.* (Wasowicz *et al.* 1993) using 1,1,3,3-tetra-ethoxy-propane as a standard. Tissue samples (50 µl) were introduced into a tube containing 29 mM/L 2- thiobarbituric acid (TBA), acetic acid (8.75 M/L) and samples were placed in a water bath and heated for 1 h at 95–100 °C. After the samples had cooled, 25 µl of 5 M/L HCl were added and the reaction mixture was extracted by agitation for 5 min with 3.5 ml n-butanol. After centrifugation, the butanol phase was separated and the fluorescence of the butanol extract was measured in a spectrofluorometer (Shimadzu RF-5500, Kyoto, Japan) using wavelengths of 525 nm for excitation and 547 nm for emission. The results are given as nmol/g protein.

Assay of SOD activity: SOD activity was assayed by the method described by Misra and Fridovich (Misra and Fridovich 1972). Supernatants were added to reaction mixture (550 µl HCO3 buffer, 400 µl EDTA, 500 µl epinephrine) and SOD activity was measured at 480 nm. Adrenochrome was

generated with epinephrine autooxidation. Since the production of adrenochrome is inhibited by Cu/Zn SOD, Cu/Zn SOD is responsible from the change of absorbance at 480 nm at which the absorbance of adrenochrome is maximum. SOD activity was expressed as the amount of the SOD standard showing activity equivalent to the determined activity. Data was expressed as U/g protein.

Assay of GSH-Px Activity: GSH-Px activity was measured by the method of Paglia and Valentine (Paglia and Valentine 1967). GSH-Px activity was measured indirectly by the coupled reaction with glutathione reductase. Oxidized glutathione (GSSG), produced upon reduction of *t*-butyl hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm that is measured spectrophotometrically. One unit of GPx activity is defined as the amount of enzyme that will cause the oxidation of 1_mol of NADPH to NADP+ per minute. Data are expressed as U/g protein.

Assay of CAT activity: Catalase activity was assayed by the method of Aebi (Aebi 1987), with hydrogen peroxide as the substrate. The final volume of each enzyme assay was 1.5 ml containing 0.5 ml of 30 mM hydrogen peroxide and 1.0 ml supernatant of tissue. Assay was performed at 25^{o}C and 240 nm. Enzyme activity was expressed as k/g protein (k: rate constant of the first order reaction as defined by Aebi).

Nitrite Assay: Nitrite levels were measured by a flourometric assay defined by Misko *et al.* (Misko *et al.* 1993). This assay is based on the reaction of nitrite with an acid form of 2,3-diaminonaphthalene to form the highly fluorescent product 1-(H)-naphthotriazole. 100 µl supernatant was mixed with 20 µl 2,3-diaminonaphtalene (DAN) in 0.62 M HCl. This mixture was incubated in the dark temperature for 10 min. The reaction was stopped by the addition of 0.28 M NaOH. The fluorescence was measured using Perkin Elmer LS 50B fluorimeter, with the excitation wavelength set at 365 nm and the emission wavelength set at 450 nm. Nitrite values are expressed as nmol/g protein.

Nitrate Assay: Nitrate levels were measured by the method of Bories *et al.* (Bories *et al.* 1995). Tissue samples (100 µl) were introduced into a tube containing 250 µL of 100 mmol/L potassium phosphate buffer (pH 7.5), 50 µl of distilled water, 50 µl of 0.2 mmol/L FAD, and 10 µl of 12

mmol/L β-NADPH. This mixture equilibrated to 25°C. To start the enzymatic reaction added 40 µl of 500 U/L nitrate reductase, immediately mixed it, and allowed the reaction to develop in the dark because of the photolability of FAD. After 45 min, the absorbance at 340 nm was recorded by spectrophotometer. Nitrate values are expressed as nmol/g protein.

Determination of Proteins: The amount of protein in the tissues was determined by using Lowry's method (Lowry et al. 1951) referring to the albumin as standard.

Statistical Analysis: The statistical software package SPSS 10.0 for windows was used to analyze the data. Number of rats was six for all groups studied. Statistical analysis was undertaken by using the non-parametric Mann–Whitney U-test. Differences were considered significant if $p<0.05$. Results were expressed as means ± standard errors.

RESULTS

In the present study, body weight and food intake were not significantly affected by exposure to stress and/or LA.

TBARS, nitrite and nitrate values were presented in Figure 1, Figure 2 and Figure 3, respectively. The data obtained from experimental groups on antioxidant enzymes are summarized in Table 1 and Table 2.

Brain and retina TBARS levels were significantly increased in the S group with respect to the control group. LA reduced retina and brain TBARS levels in the L and LS groups compared with their corresponding control groups.

Restraint stress significantly decreased brain and retina GSH-Px and CAT activities in comparison with the control group. In the stress condition, Cu,Zn-SOD activity was also decreased in the brain, but not in the retina with respect to the C group. LA increased brain and retina Cu, Zn-SOD and GSH-Px activities of the L group, whereas it decreased CAT activities of this group compared with the C group. LA increased all enzyme activities in both brain and retina in the LS group compared with the S group.

In the stress group as seen in Fig 2 and Fig 3, there was a marked increase in the brain and retina nitrite and nitrate levels in comparison with the C group. LA produced a significant decrease in brain and retina nitrite and nitrate levels of the L and LS groups with respect to their corresponding control groups.

DISCUSSION

The activities of SOD, GSH-Px and catalase were decreased while the level of TBARS was increased in the stressed rats as compared to the control group. The observed increase in lipid peroxidation in brain and retina, as determined by the measurement of TBARS, are also in agreement with previous studies (Liu *et al.* 1996, Liu and Mori 1999, Matsumoto *et al.* 1999, Yargicoglu *et al.* 2003). Additionally, the present study provides experimental data showing that brain and retina GSH-Px and CAT together brain SOD activities became decreased in the restraint stress. Therefore, in line with results of earlier reports, it could be suggested that the elevated lipid peroxidation in stress could be resulted from the decreased activities of antioxidant enzymes (Zaidi *et al.* 2003, Zaidi and Banu 2004).

There have been many reports suggesting that free radicals play an aberrant role in the mechanism of stress (Liu and Mori 1999, Olivenza *et al.* 2000, Shaheen *et al.* 1993, Kovacs *et al.* 1996, Yargicoglu *et al.* 2003, Yargicoglu *et al.* 2004). High lipid content in retina and brain plays an important role for the high vulnerability of these tissues to oxidative stress. This is because peroxidative damage of membrane lipids leads to many damages in a cell such as decreases in membrane fluidity, elevated sensitivity to oxidant stress and changes in enzyme activities (Liu *et al.* 1996, Liu and Mori 1999, Kovacs *et al.* 1996, Inoue *et al.* 1993). Therefore from the results obtained, the important consequences of chronic stress could be attributed to stress-induced lipid peroxidation. In this regard, LA was used in the present study since it was recently gained considerable attention as an antioxidant.

The treatment of rats with LA showed an increase in the activities of antioxidant enzymes with a decrease in lipid peroxidation. In accordance with the earlier studies (Marangon *et al.* 1999,

Hagen *et al.* 1999, Han *et al.* 1997, Scott *et al.* 1994, Arivazhagan *et al.* 2000, Sahin *et al.* 2006), LA has been observed to be highly effective in protecting stress-induced lipid peroxidation. Moreover, LA treatment of rats resulted in an increase in the activities of SOD, GSH-Px and CAT in the stressed and non-stressed rats with respect to their corresponding groups. From the results obtained, it could be concluded that this compound has an excellent antioxidant activity.

Our data indicated that stress induced a significant inhibition of SOD. The possible reason for this finding could be the decreased of this enzyme caused by enhanced lipid peroxidation in the stress condition (Chaudiere and Ferrari-Iliou 1999). On the other hand, declined SOD activity in brain tissues of stressed rats was brought back to a normal level with administration of LA. This result can be explained by increasing effect of LA on nerve growth factor (NGF). NGF provides expression of superoxide dismutase gene which is a factor leading to increment of SOD (Nistico *et al.* 1992).

The stress-induced decrease in the activity of GSH-Px documented in our study is corroborated by earlier investigations (Al-Qirim *et al.* 2002, Yargicoglu *et al.* 2003, Zaidi and Banu 2004). GSH-Px activity in the retina and brain of stressed rats was also brought back to a normal level with administration of LA. GSH-Px catalyzes the reduction of hydrogen peroxide (H_2O_2) to H_2O and O_2 at the expense of glutathione (GSH) (Arivazhagan *et al.* 2000, Devi and Kiran 2004). Therefore, the increment of GSH-Px activity in LA groups indicated that LA increased intracellular glutathione levels. Since GSH has an important role in the protection of vitamin E and C levels (Fang *et al.* 2002), it could be suggested that this effect of LA is one of the important antioxidant properties.

LA increased CAT activities in the brain and retina tissues. The observed decrease in the activity of CAT in stress is consistent with earlier studies (Al-Qirim *et al.* 2002, Yargicoglu *et al.* 2003, Zaidi and Banu 2004). The decline in CAT can be attributed to ineffective scavenging of H_2O_2 resulting in increased H_2O_2 levels, which can react with $O_2^{\cdot -}$ to give $OH^{\cdot}$ radical and thus increased lipid peroxidation. Catalase requires NADPH for its regeneration from its inactive form (Packer *et al.* 1997, Arivazhagan *et al.* 2000). LA is able to increase glucose uptake in vitro

(Haugaard and Haugaard 1970). Enhanced glucose uptake by cells serves as a fuel for both the pentose phosphate shunt and oxidative phosphorylation, thus bringing up the cellular levels of NADPH and nicotinamide adenine dinucleotide (NADH) and thereby also enhances the activity of catalase in restraint stress (Arivazhagan *et al.* 2000).

In the present study, we found that LA contributes antioxidant defense by increasing CAT activity in the stress group, but, in contrast to this observed effect of LA in this group, it was observed that LA significantly decreased levels of brain and retina CAT enzyme in the L group compared with the control group. The cause of these contradictory data is unclear at present and further research is needed to provide the answer.

One of the reasons for the stress-induced enhancement of free radicals may be the elevation of nitric oxide (NO) production (McCann 1997, Matsumoto *et al.* 1999). This is further supported by the present determination of nitrite and nitrate levels, which revealed a significant increase in brain and retina NO levels in restrained stressed rats. Previous studies suggested that elevated NO during stress may trigger a lipid peroxidation reaction in the brain and retina, probably via being converted to peroxynitrite (McCann *et al.* 1998, Matsumoto *et al.* 1999). Peroxynitrite in pure form will cause oxidative damage to protein, lipid, carbonhydrate, DNA, subcellular organelles and cell systems (Rubbo *et al.* 1994). On the other hand, our data on nitrite and nitrate levels of the experimental groups in the current study clearly showed that LA decreases nitric oxide production (Harnett *et al.* 2002, Packer *et al.* 1997, Haramaki *et al.* 1997). This decrement was due to direct interactions with NO, to decreased induction of inducible NO synthase, or to increased vitamin E level. Consequently, LA prevents free radical formation via increasing vitamin E, since vitamin E has been shown to be an effective antioxidant inhibiting NO-induced lipid peroxidation in rats (Siu *et al.* 1999, Escames *et al.* 1997). Therefore, it could be concluded that LA has protective effect against free radical formation thereby preventing the accumulation of toxic NO metabolites.

Finally, our present study indicated that (1) chronic stress results in significant alterations in antioxidant enzymes (2) LA is an ideal antioxidant candidate for the prevention of stress-induced

lipid peroxidation. Because of its ability to act as an antioxidant in fat- and water-soluble tissues in both its oxidized and reduced forms, LA is could be used as a potentially effective therapeutic agent in clinical conditions associated with free radical damage.

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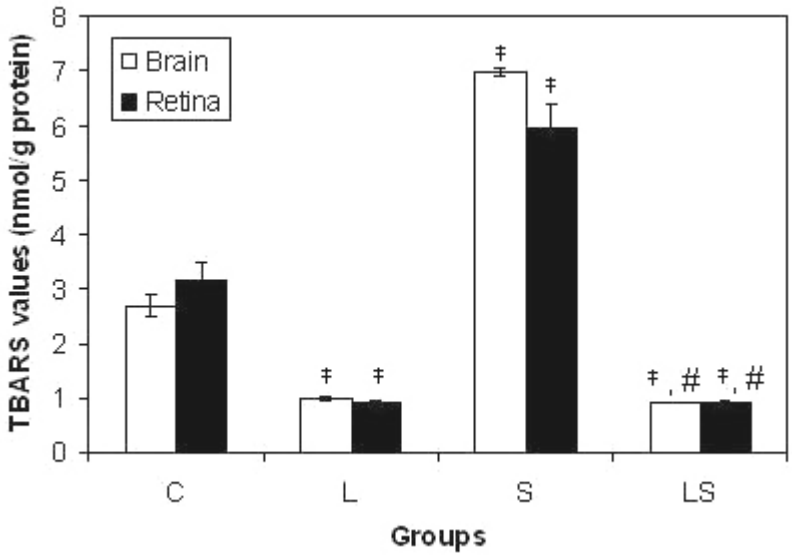

Figure 1: Thiobarbituric acid reactive substances (TBARS) values of brain and retina tissues were seen in all groups. Values are means ± standard errors. ‡: $p<0.01$ versus C group, #: $p<0.01$ versus S group. Control (C), Lipoic acid (L), Restraint stress (S), Restraint stress + Lipoic acid (LS)

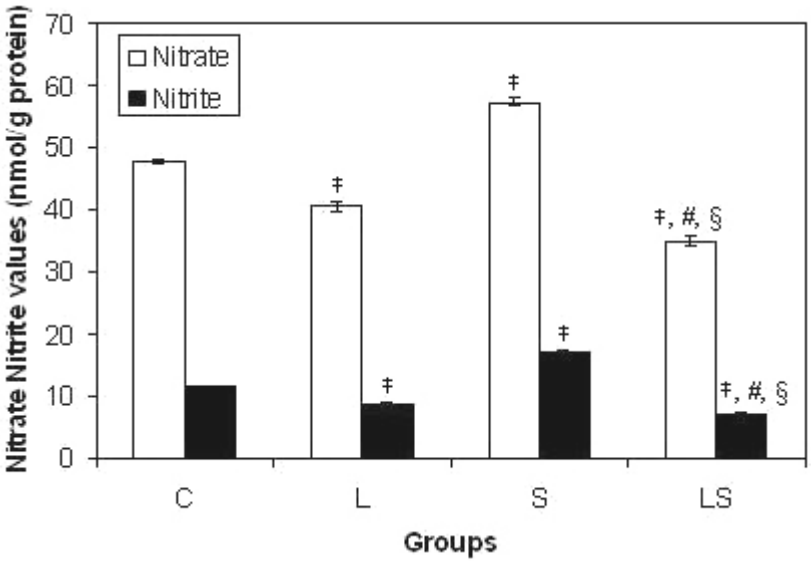

Figure 2: Nitrate and nitrite values of brain tissues were seen in all groups. Values are means ± standard errors. ‡: $p<0.01$ versus C group, #: $p<0.01$ versus S group, §: $p<0.01$ versus L group. Control (C), Lipoic acid (L), Restraint stress (S), Restraint stress + Lipoic acid (LS)

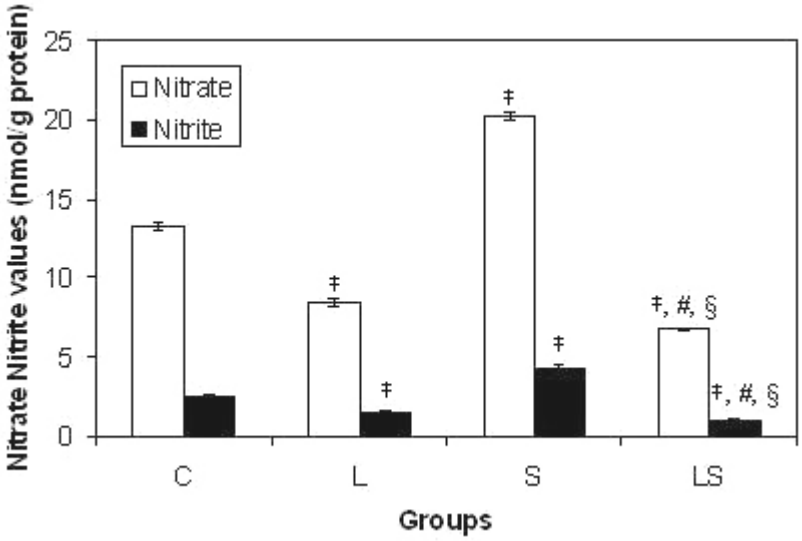

Figure 3: Nitrate and nitrite values of retina tissues were seen in all groups. Values are means ± standard errors. ‡: $p<0.01$ versus C group, #: $p<0.01$ versus S group, §: $p<0.01$ versus L group. Control (C), Lipoic acid (L), Restraint stress (S), Restraint stress + Lipoic acid (LS)

Table 1: Superoxide dismutase (SOD), Glutathione peroxidase (GSH-Px), and Catalase values of brain tissues. Values are means ± standard errors. *: p<0.05, **: p<0.01, ***: p=0.001

Groups	SOD (U/g protein)	GSH-Px (U/g protein)	Catalase (k/g protein)
Control (C)	7.89 ± 0.36	40.26 ± 0.96	3.91 ± 0.24
Lipoic Acid (L)	16.51 ± 4.36 C*	50.44 ± 0.59 C**	1.86 ± 0.25 C**
Restraint Stress (S)	3.68 ± 0.35 C***	30.45 ± 0.67 C**	1.50 ± 0.07 C**
Restraint Stress + Lipoic Acid (LS)	17.63 ± 2.34 C*, S***	40.35 ± 1.59 L**, S**	2.17 ± 0.19 C**, S*

Table 2: Superoxide dismutase (SOD), Glutathione peroxidase (GSH-Px), and Catalase values of retina tissues. Values are means ± standard errors. *: $p<0.05$, **: $p<0.01$

Groups	SOD (U/g protein)	GSH-Px (U/g protein)	Catalase (k/g protein)
Control (C)	19.53 ± 1.62	4.39 ± 0.07	51.34 ± 2.43
Lipoic Acid (L)	32.95 ± 2.69 C**	5.18 ± 0.09 C**	23.27 ± 1.51 C**
Restraint Stress (S)	20.24 ± 1.44	3.61 ± 0.10 C**	12.30 ± 0.82 C**
Restraint Stress + Lipoic Acid (LS)	27.31 ± 1.09 C*, S*	4.13 ± 0.15 L**, S**	25.92 ± 1.36 L**, S**