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Effects of systemic leptin administration on liver and plasma lipid peroxidation in cold restrain stress

[Soğuk hareketsizlik stresinde sistemik leptin uygulamasının karaciğer ve plazma lipid peroksidasyonu üzerine etkileri]

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ABSTRACT

Aim: Oxidative stress is stated to be an important mechanism of cold immobilization stress leading to tissue injury. The aim of this study was to investigate the effects of exogenous leptin administration on plasma and hepatic tissue lipid peroxidation and antioxidant status in cold-restraint stress.

Material and method: In this study Wistar albino rats were divided into four groups: 1. Control, 2. Stress, 3. Leptin (10 μg/kg leptin, i.p. twice a day, for 7 days), 4. Leptin + stress. At the end of 7th day Stress and Leptin + stress groups were exposed to cold-restraint stress, inflicted by placing the animals in a refrigerator at 4.0° ± 0.5° C for 4 h. At the end of the experiments animals were killed under thiopental sodium (50mg/kg) anesthesia. Plasma and liver malondialdehyde, glutathione and total nitric oxide levels were measured.

Results: Cold-restraint stress increased plasma and liver malondialdehyde levels whereas decreased glutathione and total nitric oxide levels. Pretreatment with leptin significantly lowered malondialdehyde levels and elevated glutathione and total nitric oxide levels.

Conclusions: Acute cold stress may lead to oxidative stress by increasing the lipid peroxidation while depleting the antioxidant capacities. Preteatment with leptin exerted a protective effect on plasma and liver against cold restraint stress induced tissue injury, probably through increasing nitric oxide content.

Key Words: Cold-restraint stress, leptin, lipid peroxidation, glutathione, nitric oxide

ÖZET

Amaç: Oksidatif stres, soğuk hareketsizlik stresinin neden olduğu doku hasarındaki önemli mekanizmalardan biridir. Çalışmanın amacı soğuk hareketsizlik stresinde eksojen leptin uygulamasının plazma ve karaciğer dokusunda lipid peroksidasyonu ve antioksidan durum üzerine etkilerinin incelenmesidir.

Gereç ve Yöntem: Çalışmada Wistar albino sıçanlar dört gruba ayrıldı: 1. Kontrol, 2. Stres, 3. Leptin (10 μg/kg leptin, i.p., günde iki defa, 7 gün süre ile) 4. Leptin+stres. 7. günün sonunda Stres ve Leptin+stres grupları ortam sıcaklığı 4.0° ± 0.5° C olan buzdolabında 4 saat süre ile soğuk-hareketsizlik stresine maruz bırakıldılar. Deneylerin sonunda hayvanlar tiyopental sodyum (50mg/kg) anestezisi altında feda edildiler. Plazma ve karaciğerde malondialdehid, glutatyon ve total nitrik oksit düzeyleri ölçüldü.

Bulgular: Soğuk hareketsizlik stresi plazma ve karaciğerde malondialdehit düzeyini arttırırken, glutatyon ve total nitrik oksit düzeylerinde azalmaya neden oldu. Leptin ile yapılan ön tedavi malondialdehit düzeylerini önemli ölçüde düşürürken, glutatyon ve total nitrik oksit düzeylerini de önemli ölçüde yükseltti.

Sonuç: Akut soğuk stres lipid peroksidasyonu arttırıp, antioksidan kapasiteyi baskılayarak oksidatif strese neden olabilir. Leptin ile yapılan öntedavi plazma ve karaciğerde soğuk hareketsizlik stresinin neden olduğu doku hasarına karşı koruyucu bir etki oluşturmuştur. Bu etki artan nitrik oksit düzeyiyle ilişkili olabilir.

Anahtar Kelimeler: soğuk hareketsizlik stresi, leptin, lipid peroksidasyonu, glutatyon, nitrik oksit

Introduction

Stress is defined as a condition in an organism that results from the action of several stressors that may be internal or external origin (1). It is well known that cold exposure may be reflected in an elevated metabolic rate and also increased production of reactive oxygen species (ROS) (2). When ROS production exceeds the capacities of protection and repair mechanisms oxidative stress occurs, resulting in a damage to proteins and lipids (3). One the most important damaging effect of free radicals on tissues is lipid peroxidation. It has been demonstrated in some forms of stress, such as exercise, starvation, cold and water-immersion restraint stress increasing the free radical generation and lipid peroxidation (4). Lipid peroxidation can be evaluated by the measurement of malondialdehyde (MDA) levels (5). Glutathione (GSH) is one of the important endogen antioxidants and apart from scavenging free radicals; also plays a role in the reduction of various disulfide linkages and maintenance of proteins in proper oxidized-reduced state (6). Acute cold stress significantly decreased blood GSH levels and perturbation of GSH metabolism in several visceral organs (7).

Leptin, the 16 kDa product of *ob* gene, is a cytokine-like molecule. Increasing number of evidences suggest that leptin apart from the regulation of food intake and energy homeostasis, participates in many physiological functions including regulation of neuroendocrine and immune systems, reproduction, angiogenesis and lipolysis (8).

There have been controversial reports about the oxidant or antioxidant roles of leptin in different tissues. It is well known that leptin plays an important role in gastric mucosal integrity and gastroprotection (8). However, there is some evidence indicating the correlation of serum leptin levels with hepatic fibrosis but not with hepatic steatosis or inflammation (9). It is also known that leptin stimulates endothelial nitric oxide (NO) production (10) and intravenous injection of leptin increases the plasma nitrite/nitrate concentration in a dose-dependent manner in normotensive rats (11).

Some studies reported that NO is an important mediator of hepatotoxicity and the high production of NO causes tissue injury (12, 13). However, in some models of inflamation, it has been shown that inhibition of NO increases tissue dysfunction or injury (14). Consequently, the pro-oxidant and/or anti-oxidant role of NO seems to be controversial. Although several studies have investigated the effects of cold-restraint stress on the antioxidant system and induction of lipid peroxidation in several tissues, no information is available regarding the antioxidant effect of systemic leptin on cold restraint stress induced plasma and hepatic tissue injury so far. In the present study liver was preferred for its high metabolic rate.

Thus, in the present study the effects of exogenous leptin administration on cold-restraint stress induced plasma and hepatic tissue damage were examined by evaluation of MDA, GSH and total nitric oxide (NO) levels.

Material and Methods

Animals and Study Design

The following experiments were approved by the Ethical Committee of Gazi University for the care and use of laboratory animals. 32 male Wistar albino rats weighing 200±20 g were used in this study. They were fed a standard laboratory diet and tap water ad libitum and kept in a room with controlled temperature (22 ± 1^{o} C), and a 12:12-h light-dark cycle. They were divided into four groups each consisting of 8 animals and allowed free access to standard diet and water ad libitum.

1. Control group; physiological saline solution, PS, i.p. twice a day, for 7 days. 2. Stress group ; PS, i.p, twice a day, for 7 days. At the end of the 7th day rats were exposed cold-restraint stress. 3. Leptin group ; 10 µg/kg, recombinant rat leptin (CALBIOCHEM), i.p. twice a day, for 7 days. (8). 4. Leptin + stress group; 10 µg/kg leptin, i.p. twice a day, for 7 days. At the end of the 7th day rats were exposed cold-restraint stress. Animals were fasted for 24 h before the stress induction, but have had free access to water. A cold-restraint stress model was inflicted by placing the animals in a refrigerator at 4.0^{o} ± 0.5^{o} C for 4 h (15). Control and leptin groups were not exposed to stress. At the end of the experiments animals were killed under thiopental sodium (50mg/kg) anesthesia. Plasma was separated and stored. The liver was immediately excised and kept in liquid nitrogen and stored at –70^{o} C until subsequent assay.

Biochemical determinations

Determination of plasma lipid peroxide level

Plasma lipid peroxide levels were estimated by the method of Kurtel et al (16). Briefly, lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS). Lipid peroxide level was expressed in terms of MDA equivalent using an extinction coefficient of 1.56 x 10^{5} M^{-1} cm^{-1} (17).

Determination of plasma RSH level

The RSH levels were determined by the method of Kurtel et al. (16). The RSH levels were calculated assuming a molar extinction coefficient of 13.000 mol^{-1} cm^{-1} at 412 nm.

Determination of tissue MDA and GSH levels

Hepatic tissue samples were obtained and frozen immediately in liquid nitrogen, than kept at –70^{o} C until analyses were performed. Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) (18). Lipid peroxide levels are expressed in terms of MDA equivalents using

an extinction coefficient of 1.56 x 10^5 mol^{-1} cm^{-1}.

The GSH levels were determined by a modified Ellman method (19). The GSH levels were calculated using an extinction coefficient of 13,600 mol^{-1} cm^{-1} .

Measurements of MDA, GSH and RSH were carried out at room temperature using a spectrophotometer (UV 1208, Shimadzu, Japan).

Determination of tissue and plasma NO levels

NO levels were measured with a Elisa reader by vanadium chloride (VCl_3) / Griess assay. Prior to NO determination the tissues were homogenized in five volumes of phosphate buffer saline (pH=7) and centrifuged at 2000 g for 5 min. 0.25 ml 0.3 M NaOH was added to 0.5 ml of the supernatant. After incubation for 5 min. at room temperature, 0.25 ml of 5% (w/v) $ZnSO_4$ was added for deproteinization. This mixture was then centrifuged at 14 000rpm for 5 min and supernatants were used for the assays (20). Nitrate standard solution was serially diluted. After loading the plate with samples (100 µl), addition of vanadium III chloride (VCl_3) (100 µl) to each well was rapidly followed by addition of Griess reagents, sulphanilamide (SULF) (50 µl) and N-(1-naphtyl) ethylenediamide dihyrochloride (NEDD) (50 µl). After the incubation (usually 30-45 min), samples were measured at 540 nm using an ELISA reader. The same procedure was applied to the plasma samples after deproteinization was carried out.

Statistical Analysis

Data are presented as means ± S.D. Statistical analysis using by SPSS 13.0 pack for Windows. Values of $p<0.05$ were regarded as significant. First of all, for NO, GSH/RSH, and NO levels in liver and plasma, Kolmogorov-Smirnov test was used to display whether the data distributed normally or not. Data for those three parameters was normally distributed in both liver and plasma. Then ANOVA test was employed for variance analysis, and there was a statistically significant difference between plasma and liver ($p<0.05$). Followed by ANOVA test, Levene test was employed for the equal variance of each variable (MDA, GSH, NO) in four groups. Results of this test were displayed below:

a. In liver Tissue: MDA and NO levels have a homogenous distribution, and Tukey Multiple Comparison test was used for pairwise comparisons. Because of GSH levels showed non- homogenous distribution, Tamhane test was employed

b. In plasma: MDA, RSH, and NO levels have a homogenous distribution, and Tukey Multiple Comparison test was used for pairwise comparisons.

Results

Plasma MDA levels are shown in Figure 1. being 2.56±0.02 nmol/ml, 3.07±0.05 nmol/ml, 2.37±0.02 nmol/ml and 2.6±0.02 nmol/ml in control, stress, leptin and leptin + stress groups respectively. Plasma MDA levels were significantly higher ($p<0.05$) in the stress group than the control group. Plasma MDA levels were significantly lower in the leptin group comparing all the other three groups ($p<0.05$). Leptin administration (10µg/kg i.p. for 7 days) prior to cold-restraint stress led to a significant decrease in plasma MDA levels as compared to the stress group ($p<0.05$). The difference were not statistically significant and a similar result was obtained between the control and the leptin+stress groups.

Plasma RSH and NO levels are shown in Figure 2. and Figure 3. respectively. Plasma RSH levels were 145±2.9 nmol/ml, 92.62±2.29 nmol/ml, 147.5±1.7 nmol/ml and 132.8±1.7 nmol/ml in control, stress, leptin and leptin + stress groups respectively. Plasma NO levels were 44.36 ± 1.17 µmol/l, 26.75± 0.81 µmol/l, 51.13± 1.41 µmol/l and 38.38± 0.92 µmol/l in control, stress, leptin and leptin + stress groups respectively. Plasma RSH and NO levels were decreased in after cold-restraint stress ($p<0.05$). In comparison to the control group, systemic leptin administration increased the NO levels ($p<0.05$), but there was no significant change in RSH levels (Group 3). Pretreatment with i.p. leptin resulted in a significant increase in plasma RSH and NOx levels (Group 4) when compared with the results measured in that group which was subjected to cold-restraint stress alone (group 2) ($p<0.05$, $p<0.05$ respectively).

The results about mean liver MDA levels are shown in Figure 4. Tissue MDA levels were 46.68±0.89 nmol/g, 52.75±1 nmol/g, 39.35±0.57 nmol/g and 47.37±0.81 nmol/g tissue in control, stress, leptin and leptin + stress groups respectively. Tissue MDA levels was found to be higher in the stress group when compared with the control group ($p<0.05$). The most significant decrease in MDA levels were seen in leptin group ($p<0.05$). Exogenous leptin significantly decreased MDA levels in the liver of cold-restraint stress treatment rats (Group 4, $p<0.05$). The difference was not statistically significant between the control and the leptin + stress groups. Liver GSH and NO levels are shown in Figure 5. and Figure 6. respectively. Tissue GSH levels were 5.62±0.12 µmol/g, 4.11±0.05 µmol/g, 6±0.04 µmol/g and 4.61±0.1 µmol/g tissue in control, stress, leptin and leptin + stress groups respectively. Tissue NO levels were 23.73±1 µmol/g, 18.56±0.49 µmol/g, 27.62±0.48 µmol/g and 21.79±0.81 µmol/g tissue in control, stress, leptin and leptin + stress groups respectively. GSH and NO levels in the liver was significantly lower in stress group as compared with the control group ($p<0.05$, $p<0.05$ respectively). Leptin administration prior to cold-restraint stress (group 4) led to a significant increase in liver GSH and NO levels as compared to the stress group ($p<0.05$, $p<0.05$ respectively). The most significant increase in NO level was seen in the leptin group (Figure 6).

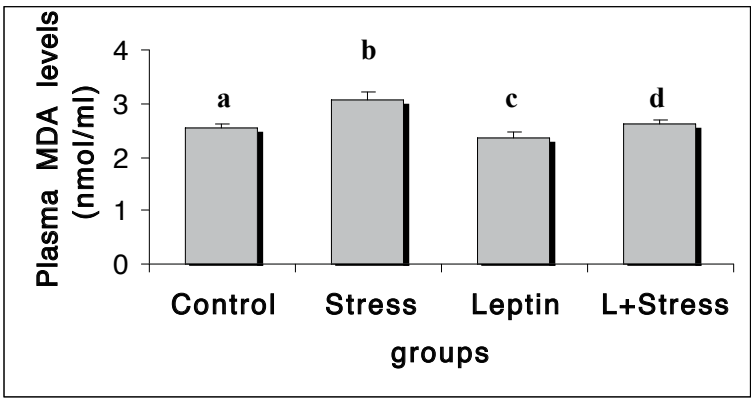

Figure 1. MDA levels in the plasma of control, stress, leptin and leptin+stress groups.
Each value represents the mean ± S.D. of eight animals per group.
$p<0.05$: a-b, a-c, b-c, b-d c-d

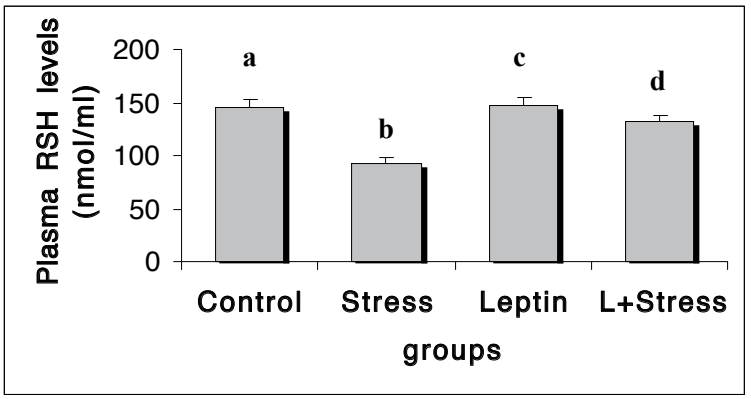

Figure 2. RSH levels in the plasma of control, stress, leptin and leptin+stress groups.
Each value represents the mean ± S.D. of eight animals per group.
$p<0.05$: a-b, a-d, b-c, b-d, c-d

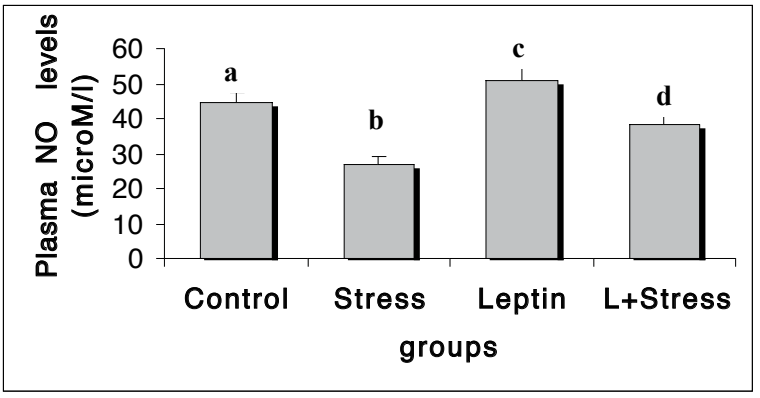

Figure 3. NO levels in the plasma of control, stress, leptin and leptin+stress groups.
Each value represents the mean ± S.D. of eight animals per group.
$p<0.05$: a-b, a-c, a-d, b-c, b-d, c-d.

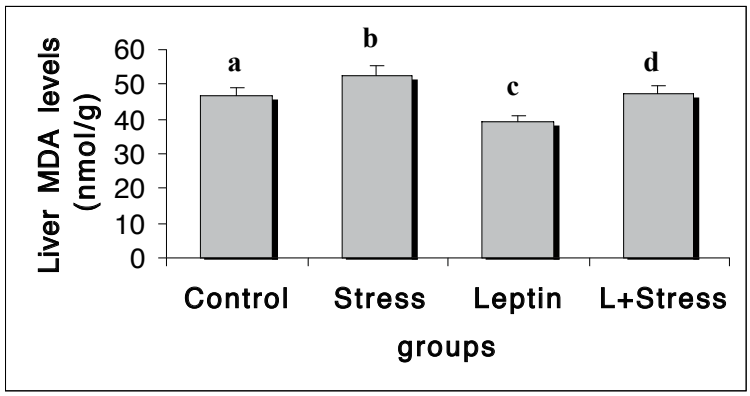

Figure 4. MDA levels in the liver of control, stress, leptin and leptin+stress groups. Each value represents the mean ± S.D. of eight animals per group. $p<0.05$: a-b, a-c, b-c, b-d, c-d.

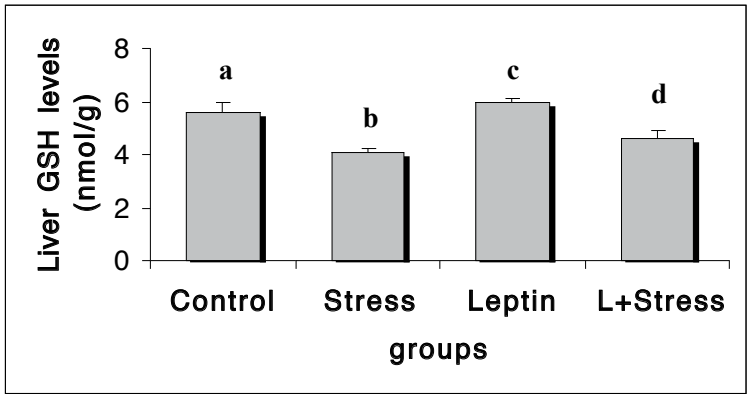

Figure 5. GSH levels in the liver of control, stress, leptin and leptin+stress groups. Each value represents the mean ± S.D. of eight animals per group. $p<0.05$: a-b, a-d, b-c, b-d, c-d.

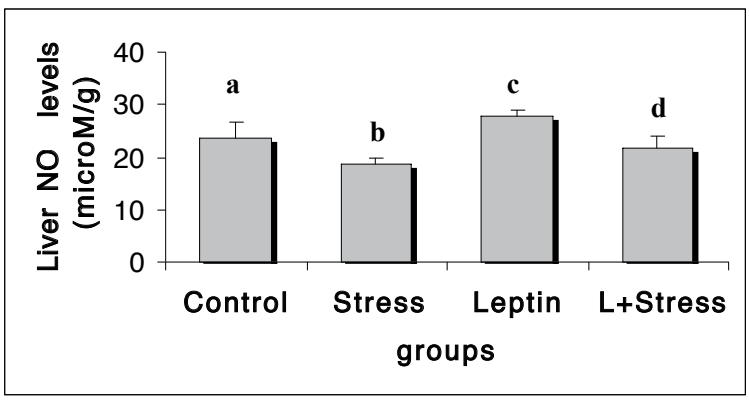

Figure 6. NO levels in the liver of control, stress, leptin and leptin+stress groups. Each value represents the mean ± S.D. of eight animals per group. $p<0.05$: a-b, a-c, b-c, b-d, c-d.

Discussion

The physiological components of stress response to cold covers metabolic, circulatory and hormonal process; however, the cellular and molecular mechanisms mediating these responses remains to be elucidated. Immobilization and acute cold stress are widely used experimental model accompanied by considerable decrease in antioxidative capacity in animals (21, 22).

Lipid peroxidation has been implicated in a number of deleterious effects and increase in the levels of TBARS indicate the enhanced lipid peroxidation leading to tissue injury and failure of antioxidant defense mechanisms to prevent the formation of excess free radicals (23). In the present study liver and plasma MDA levels were found to be significantly increased in the rats exposed to restraint-cold stress when compared to the controls. These results are in agreement with the previous findings which are related to stress-induced lipid peroxidation in plasma and liver of the animals (22, 24). Our findings showed that the antioxidant system was also affected by cold-restraint stress. GSH is an important endogenous defense substance against the reactive oxygen species (ROS) and the tissue GSH concentration reflects a potential detoxification marker. It has been previously reported that cold stress reduced the GSH levels in liver and in the other tissues in mice (21) and rats (25). Shustanova et al. have reported that cold stress caused an inhibition of antioxidant enzyme activities such as glutathione reductase in brain, liver and erythrocytes (26). These results are in accordance with the results of the present study, since the cold-restraint stress resulted in an activation of free radical accompanied by an accumulation in liver and plasma of lipid peroxidation products and a decrease of endogenous antioxidant marker GSH. However, the increase in plasma GSH level might be due to the penetration of tissue GSH to plasma. According to the results of the present study, it can be assumed that the negative relation between MDA and GSH levels in in the liver and plasma of the rats in the stress group may be the evidence of the susceptibility of the proteins to oxidation.

It has been well known that NO radicals have a direct antioxidant effect through their reaction with free radicals and iron-oxygen complexes. In addition to serving as a stabilizer and carrier of NO, S-nitrosoglutathione (GSNO) may have protective effects through transnitrosylation reactions. Moreover, it has been suggested that NO and GSNO act like a free radical scavenger at moderate concentrations which are 50-100 times more potent than that of GSH (27). Peralta et al. reported that mtNOS activity and expression decreased in liver and skeletal muscle during the first 10 days of the cold exposure (28). The positive correlation between cold stress and reduced NOS activity was supported by the findings of Zhu et al. (29), Lee et al. (30) in different tissues.

In the present study we observed that NO activity both in the plasma and liver was found to be significantly decreased during cold-restraint stress. These results are also in accordance with the findings that indicate the development of resistance to cold stress might be abolished by decreasing NO (31). The decrease of NO synthesis suggests an inability of the cells to synthesize GSH.

According to the results of the present study, leptin pretreatment significantly reduced the cold-immobilization induced increase in MDA levels of plasma and liver. Although leptin administration to normal rats did not change the plasma and liver MDA and GSH levels, cause an increase in plasma and tissue GSH levels obtained from restraint-cold stress induced rats. There are controversial reports about the prooxidant or antioxidant roles of leptin in different tissues. This discrepancy might be due to the different experimental methods used and species differences. In some studies leptin administration, increased lipid peroxidation and inhibited antioxidant system in mice brain (32). But it has been shown that leptin , decreased TBARS level of mice liver, heart and kidney (33), exerted a potent gastroprotective effect against ischemia-reperfusion (34) or ethanol induced stomach damage (35), suppressed apoptosis in gastric mucosa (15) and liver (36) . In this study leptin caused an increase in GSH, while decreased MDA levels both in plasma and liver tissue which were found to be decreased and increased respectively obtained from rats exposed to restraint-cold stress.

Recently it has been shown that leptin causes an increase in endothelial NO production (37) and contributes to the healing process of peptic ulcer by stimulating local production of NO (8) Our results are in accordance with these findings. In the present study we observed that leptin causes an increase in plasma and tissue levels of NO in normal rats and also causes an increase in the levels of NO which is found to be decreased during restraint-cold stress.

These results indicate that restraint-cold stress causes an increase in MDA levels and decrease NO levels in plasma and liver tissues, decrease GSH level in tissue while increase in plasma. Leptin administration causes an increase both in decreased GSH and NO levels and decreases the increment of MDA levels.

In conclusion it can be assumed that stress affects the progression of several disease via the generation of ROS and inhibition of antioxidant system as well as NO synthesis. Leptin probably by increasing NO synthesis prevents the increase of MDA and decrease in GSH levels via antioxidant and tissue protective effects.

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