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Immobilization stress-induced changes in liver function, oxidative balanceand inflammatory responses ofwistar albino rats

Omer M. Ahmed[1], Amel O. Bakhiet[2*] and Khalid A. Abdoun[3]

1. Laboratory Animals Unit, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia
2. College of Veterinary Medicine, Sudan University of Science and Technology, Khartoum, Sudan
3. Department of Animal Production, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia

[*]Corresponding author: Amel Omer Bakhiet E-mail: amel33@hotmail.com

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ABSTRACT

The present study aimed to investigate the effect of immobilization stress onliver function, oxidative damage andinflammatory responsesin male wistar albino rats. Twelve wistar albino rats were randomly divided into two groups, six rats each. One group was not exposed to immobilization stress and served as control. The other group was exposed to immobilization stress. The rats in immobilized group were exposed to immobilization stress for 4 hours daily for 6 consecutive weeks. At the end of the experiment animals were sacrificed, and the blood samples and liver tissues were collected for the estimation of biochemical markers. Liver function assessed by serum transaminase activity showed significant (P <0.05) increases in ALT and AST levels, and a significant reduction in liver protein content of immobilization stressed rats. The oxidative status measured by malondialdehyde (MDA) level in liver tissues was significantly (P < 0.05) increased in immobilization stressed rats.Whilethe antioxidant biomarkerssuperoxide dismutase (SOD), reduced glutathione (GSH), glutathione peroxidase (GPx) and catalase (CAT)levels were significantly (P <0.05) reduced in immobilization stressedrats. The pro-inflammatory cytokine biomarkers TNF-α, IL-1ß and IL-6were significantly (P <0.05) elevated, whilethe anti-inflammatory cytokine IL-10 was significantly (P <0.05) reduced in immobilization stressedrats. The obtained results indicate that immobilization stress induced oxidative damage and inflammatory response which was reflected in a compromised liver function. This indicates that immobilization stress can be used as an easy and convenient method of stress induction.

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INTRODUCTION:

Stress is usually recognized as a state of altered physiological homeostasis, and the ability to cope with such stressful stimuli is a crucial determinant of health and disease.

Immobilization/restraint stress is an easy and convenient method to induce both psychological (escape reaction) and physical stress (muscle work) resulting in restricted mobility and aggression (Singh et al., 1993; Romanova et al., 1994). Chronic psychological stress is one of the major non-genomic factors that contribute to several pathological states such as psychiatric disorders, immunosuppressant and neurological impairments. However, it is well known that intensive stress response results in the creation of reactive oxygen species (ROS), e.g. hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-) and superoxide anion radical (O_2^-) that cause lipid peroxidation, especially in membranes and can play an important role in tissue injury (Kovacs et al., 1996). One common method to determine the degree of lipid peroxidation is by measuring the level of malondialdehyde (MDA), a by-product of the lipid peroxidation process (Millan-Plano et al., 2003; Topal et al., 2004). Additionally, measuring changes in the concentration of reduced glutathione (GSH), an endogenous antioxidant, has also been used as an indicator of lipid peroxidation severity (Abd El-Gawad and El-Sawalhi, 2004; Zaidi and Banu, 2004). ROS can lead to oxidation of proteins and DNA, peroxidation of lipids, and ultimately cell death (Butterfield et al., 2001). Lipid peroxidation produces large amounts of aldehyde, such as HNE, MDA and acrolein which contribute to membrane formation (Butterfield et al., 2002). HNE and acrolein contribute to membrane damage and cell death induced by a variety of oxidative insults (Esterbauer et al., 1991), and through alterations of protein structure is capable of inhibiting DNA, RNA and protein synthesis (Subramaniam et al., 1998). The levels of damage in RNA are usually higher than those in DNA under oxidative stress (Fiala et al., 1989; Wamer et al., 1997; Hofer et al., 2005; Shan and Lin, 2005) suggesting that RNA damage may be an important issue to cells and organisms (Bellacosa and Moss, 2003). Recently, remarkable increases in oxidative damaged RNA products were reported in patients of age-related disorders including Alzheimer's disease (Abe et al., 2002), Parkinson's disease (Zhang et al., 1999), myopathies (Tateyama et al., 2003) and atherosclerosis (Martinet et al., 2004). Furthermore, it has been reported that radiation-induced oxidative stress in animal studies had caused significant damage in hepatic RNA concentrations (Abou-Seif et al., 2003; El-Missiry et al., 2007).

Cytokines may act as classic endocrine secretion. T helper 1 (Th1) and T helper 2 (Th2) cells represent two subpopulations of CD4+ T cells which can be differentiated by their cytokine profiles. It is now recognized that the Th1/Th2 balance is important for immune-regulation (Liblau et al., 1995; Nishimura and Ohta, 1999). Th1 cells produce interferon-c (IFN-c) and IL-2, which induce differentiation of CD4+ T cells to Th1 cells and inhibit the proliferation of Th2 cells. In contrast, Th2 cells secrete interleukin-4 (IL-4), IL-6 and IL-10, which induce differentiation of Th2 cells and inhibit Th1 cells. Both Th1 and Th2 cells secrete GM-CSF and TNF-a. Th1 activation contributes to cell-mediated immunity whereas Th2 activation favors the humoral immune response (Szabo et al., 2000; Mullen et al., 2001; Das et al., 2001). Conditions associated with significant changes in glucocorticoid concentration, such as the introduction of acute or chronic stress, might affect the susceptibility or course of infection by modulating the Th1/Th2 balance (Elenkov, 2004;

Viveros-Paredes et al., 2006). Among these cytokines tumor necrosis factor-a (TNF-a) and interleukin-6 (IL-6) belong to pro-inflammatory cytokines, which are pleiotropic molecules produced by a variety of peripheral cell types as well as those cells in the central nervous system (CNS). Therefore, in the current experiment we selected certain oxidative stress and liver function biomarkers and three cytokines including IL-1β, IL-6 and TNF-α to observe the possible stress induced changes in lipid peroxidation, liver function and inflammatory responses.

MATERIALS AND METHODS:

Animals: Twelve male Wistar albino rats were received from Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The rats were maintained under controlled ambient temperature (22–24 ºC), relative humidity (50-60 %) and light (12L: 12D cycles), and kept on free access to clean tap water and pelleted rodent diet (Grain silos and flour mills organization, Riyadh, Saudi Arabia). The nutrients composition of the pelleted rodent feed used in the present study is shown in Table 1.

All experimental procedures including immobilization, euthanasia and other experimental procedures were conducted in accordance with the guide for the care and use of Laboratory Animals Institute of Health (NIH Publications No. 80-23; 1996) and approved by Ethical Committee of Experimental Animal Care Centre (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia).

Table (1): Chemical composition of rodent pelleted feed

Crude protein (%)	Crude fat (%)	Crude fiber (%)	Ash (%)	Salt (%)	Ca (%)	P (%)	Vitamin A (IU/g)	Vitamin D (IU/g)	Vitamin E (IU/g)	ME(Kcal/kg)
20	4	3	6	0.5	1	0.6	20	2.2	70	2850

Immobilization procedure: The immobilization/restraint stress model used in the present study was adopted from earlier studies (Zaidi and Banu, 2004) with slight modifications. Placing animals in the exact size tube was reported to be a good restraint procedure since it involves minimum pain with minimum movements including that of the tail (Pare and Glavin, 1986). Therefore, immobilization stress was induced by placing each animal in a plastic/well-ventilated tube of the exact animal size (Figure 1).

Figure 1: Procedure for exposingrats to immobilization stress.

Experimental design: This study was conducted using the minimum necessary number of animals (six animals for each group) in order to obtain reliable and statistically significance results for such types of experiments. Animals were randomly divided into two groups, six rats each. The experimental groups comprised of the control and the immobilized groups.The rats in immobilized group were exposed to immobilization stress for 4 hours daily, for 6 consecutive weeks. During the immobilization stress procedure, rats were deprived of food and water. At end of the experiment, rats were euthanized immediately after the last immobilization stress session. Blood samples were withdrawn through cardiac puncture into disposable centrifuge tubes and centrifuged at 3000 rpm for 10 minutes. Serum samples were separated, properly labeled and stored at -20ºC till laboratory analysis. Liver tissues were immediately excised, washed with chilled normal saline, dipped in liquid nitrogen for one minute and stored at -70ºC till laboratory analysis.

Serum biochemical analysis: ALT and AST levels in serum samples were estimated using the commercially available diagnostic kits (Randox diagnostic reagents, Randox Laboratories, USA). Whereas, IL-6, IL-1β, TNF-α, and IL-10 levels in serum samples were assayed by enzyme–linked immunosorbent assay (ELISA) kits (Shanghai SenXiong Science and Technology Company, China). The estimation protocol followed the instructions provided by the manufacturers.

Liver tissues samples preparation and analysis: Liver tissues were excised, washed with chilled normal saline, dipped in liquid nitrogen for one minute and preserved at -70°C till laboratory analysis. Liver tissues were homogenized in 50mM phosphate buffered saline (pH 7.4) by using a glass homogenizer (Omni International, Kennesaw, GA, USA). The liver tissues homogenates were then centrifuged at 1000g for 10 minutes at 4ºC, and the nuclei and unbroken cells were separated. The pellets were discarded, and the portions of the supernatants were centrifuged again at 12000g for 20 minutes, and hence post-mitochondrial supernatants were obtained.

Thiobarbituric acid reactive substance (TBARS) and glutathione (GSH) levels were estimated in liver tissues homogenates. Whereas, the activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) were measuredin post-mitochondrial supernatants.

Statistical analysis: Data were expressed as Means ± SD and analyzed using one-way ANOVA followed by Student-Newman-Keuls multiple comparisons test; six rats were used in each group.

RESULTS:

Immobilization stress resulted in a significant ($P < 0.05$) elevation of both ALT and AST serum concentrations.Whereas, the liver protein content of immobilized stressed rats was significantly ($P < 0.05$) decreased (Table 2). This suggests that immobilization stress has negatively affected livers functions.

Table (2): The effects of immobilization stress on serum AST and ALT concentrations and liverprotein content of wistar rats (Means±SD).

Parameter	Control	Immobilization stress	P value
ALT (U/L)	21.43 ±3.06	28.86 ±9,06	<0.05
AST (U/L)	19.87 ±4.84	29.65 ±5.61	<0.05

Liver protein (mg/100 mg)	13.30 ±2.27	8.85 ±1.61	<0.05

AST= Aspartate aminotransferase; ALT = Alanine aminotransferase (Number of animals = 6).

The effect of immobilization stress on lipid peroxidation was studied by investigating the changes in the level of oxidants and antioxidants biomarkers in liver tissues. Immobilization stress resulted in a significant (P <0.05) increase in malondialdehyde (MDA) concentration in liver tissue compared to control group (Table 2). While, hepatic antioxidants including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase and reduced glutathione (GSH) were significantly (P <0.05) reduced in immobilization stressed rats (Table 3).

Table (3): The effects of immobilization stress on oxidative status in liver tissues of wistar rats (Means±SD).

Parameter	Control	Immobilization stress	P value
MDA (nmol/mg protein)	2.18 ±0.46	3.89 ±0.48	<0.05
SOD (U/mg protein)	1.86 ±0.35	1.00 ±0.35	<0.05
CAT(nmol/mg protein)	10.72 ±1.97	7.37 ±1.84	<0.05
GPx(nmol/mg protein)	1.38 ±0.17	0.65 ±0.29	<0.05
GSH(nmol/mg protein)	5.25 ±0.64	4.29 ±0.26	<0.05

MDA = Malondialdehyde (Thiobarbituric acid reactive substances); SOD = Superoxide dismutase; CAT = Catalase; GPx = Glutathione peroxidase; GSH = Reduced glutathione; (Number of animals = 6).

Serum pro-inflammatory cytokines levels including TNF-α, IL-1β and IL-6showed significant (P <0.05) increase in the immobilization stressed rats group compared to the control group (Table 4).However, serum levels of IL-10 was significantly (P<0.05) decreased in immobilization stressed rats (Table 4).

Table (4): The effects of immobilization stress on inflammatory response of wistar rats(Means±SD).

Parameter	Control	Immobilization stress	P value
TNF-α (pg/mL)	84.59±19.52	127.65±27.03	<0.05
IL-1β (pg/mL)	28.75±6.90	48.31±16.34	<0.05
IL-6 (pg/mL)	63.06±21.43	79.03±21.77	<0.05
IL-10	223.91±26.25	181.65±19.97	<0.05

TNF-α = Tumor necrosis factor- alpha; IL-1β = Interleukin-1 beta; IL-6 = Interleukin-6; IL-10 = Interleukin 10; (Number of animals = 6).

DISCUSSION:

Immobilization stress was reported to be a good model for investigating the alterations occurring in oxidant–antioxidant balances in animal tissues particularly in the vulnerable organs such as brain and liver (Gumuslu et al., 2002; Sahin and Gumuslu, 2007). The results of the present study showed significant increases in liver enzymes including ALT and AST,and a significant reduction in liver protein content due to the immobilization stress. Liver transaminases (AST and ALT) are useful biomarkers of liver injury (Johnson, 1999; McClatchey and Kenneth, 2002; Mengel et al., 2005). Where, elevated transaminases (AST and ALT) may be an indicator of liver damage (Giboney, 2005).Therefore, these alterations in liver enzymes and protein content indicate that immobilization stress had negatively affected liver function.

Lipid peroxidation is the oxidative damage of cellular membranes and other lipid containing molecules under oxidative stress conditions (Nawrot et al., 2008). Therefore, we tested the oxidative – antioxidant balance in liver

tissues as an indicator of lipid peroxidation in the liver of rats. Immobilization stressedrats in the current study showed a significant increase in liver tissues malondialdehyde (MDA) concentration, and a significant reductionin antioxidants (GSH, GPx, SOD, CAT) concentrations in liver tissues. This could be attributed to the immobilization stress induced stimulation of free radicals production in the liver tissues of rats (Davydov et al., 2004).The inactivation of liver antioxidant enzymes observed in this study may ultimately result inliver lipid peroxidation (Sahin and Gumuslu, 2007). Hence, immobilization stress may play a potential role in aggravating liver diseases like hepatic inflammation via generation of reactive oxygen species (Zaidi et al., 2005).

There are two types of cytokines which include pro-inflammatory cytokines and anti-inflammatory cytokines. Pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. While, anti-inflammatory cytokines (IL-4, IL-10, IL-11, and IL-13) control the pro-inflammatory cytokine response (Zhang and An, 2007).Immobilization stress of rats used in the present study resulted in a significant elevation of pro-inflammatory cytokines and a significant reduction of anti-inflammatory cytokine IL-10. This indicates that immobilization stress may havecaused cell injury and inflammation which is reflected in the observed elevation of pro-inflammatory cytokines.

CONCLUSIONS:

Immobilization stress in rats induced liver oxidative damage and provoked inflammatory responses and thus compromised liver function. This indicates that immobilization stress can be used as easy and convenient method of stress induction in studies aiming to mitigate the negative effects of stress using different treatment strategies.

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