Effects of Dalteparin on Structure of Hippocampal Neurons of Rats in Chronic Stress

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A B S T R A C T

Introduction: Stress is defined as any environmental change that disturbs the maintenance of brain homeostasis. Stress leads to production of pro-inflammatory cytokines that provoke neurodegenerative disorders. In the present study, we investigated the effects of dalteparin on hippocampal neuronal death induced by chronic stress in rats.

Methods: the study was carried out on 60 adult male wistar rats, weighing 200-250 gr. The rats were randomly divided into three groups: control, stress and stress + dalteparin (SD) groups. Animals in the stress and stress + dalteparin group were exposed to chronic stress for 4 weeks. Animals in the stress + dalteparin (SD) group received dalteparin (70,100 and 140 IU/kg/days i.p.) during the stress period. After the last stressor animals were sacrificed and concentration of IL-6 in serum was measured using ELISA. All animals were reperfused and their brains were processed for histological analysis through Nissl analysis.

Results: We found that the serum concentration of IL-6 was significantly higher in the CMS (Chronic Mild Stress) exposure group than in the control group ($p<0.05$). Moreover, dalteparin, dose dependently decreased IL-6 concentration in the SD groups. Chronic stress also resulted in significant cell loss in hippocampal CA1, CA3 and hilus. Dalteparin markedly inhibited the decreases in number of hippocamoal CA1 and CA3 ($p<0.01$) and hilus ($p<0.05$) neurons caused by chronic stress.

Discussion: chronic stress damages hippocampal CA1, CA3 and hilus neurons, and dalteparin protects hippocampus from damage induced by chronic stress.

1. Introduction

Stress is a condition that elevates activities of physiological system and therefore, leads to disruption of body homeostasis. Chronic stress is especially a risk factor for many mental disorders such as depression (Maes, 2001; Checkley, 1996). External stressors play important roles in development of mental disorders. Stress also has strong effect on cognition and memory. Clinical research has shown that stress alters both morphology and function of hippocampus (Magarinos, 1996). The chronic mild stress induces inflammation, e.g. increased IL-1ß, IL-6, TNF-α (Maes, 1999). External stress is accompanied by an increased production of pro-inflam-

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matory cytokines that can provoke neurodegenerative disorders. Stressors, usually activate immune system, and result in different levels of inflammation both in the body and the brain (Maes, 2001). Neurodegeneration is one of the consequences of inflammatory processes in different parts of the brain.

Peripherally generated pro-inflammatory cytokines, can induce production of cytokines in the CNS and initiate brain neuro-inflammation (Kubera, 2010). For example, systemic LPS administration induces a peripheral and central inflammation resulting in neuro-degeneration (Qin, 2007). Pro-inflammatory cytokines play an important role as mediators of external and internal stressors responses (Maes, 1995; Maes, 2010). Multiple studies have shown that inflammation is accompanied by increased production of oxygen radicals, which produce a variety of adverse effects in the hippocampus and may contribute to neuronal death (Maes, 2007; Forlenza, 2006; Sapolsky, 2004). In humans, exposure to physical and psychological stress is accompanied by increased plasma level of IL-6 (Zhou, 1993; Kiecolt-Glaser, 2003). Xiu et al observed that CMS-treated rats show an increase in serum IL-6 level (Xiu, 2010). The release of pro-inflammatory cytokines contributes to neurodegeneration (Wang, 2008; Dantzer R, 2008).Wager-Smith and Markou showed that stressful life events lead to microdamage in the brain (Karen Wager-Smith, 2011). Many studies have used chronic stress protocols and shown that stress leads to structural changes in the brain (Christian, Miracle, 2011; Zhang Yan-Mei, 2003; Pablos, 2006). Previous evidence suggests that chronic stress has profound effects on structure and function of hippocampal neurons (Goshen I, 2009; Li S, 2008; Mineur, 2007). It is known that the hippocampus is very sensitive to stress stimuli in both humans and animals, which makes it vulnerable to damage during chronic stress exposure (McEwen, 1999). Many of stress-induced structural changes are transient and disappear after recovery. Reports also indicates that long term exposure to stress causes necrosis of brain tissues and morphological damage to the neurons in the hippocampus CA2 and CA3 regions (Endo Y, 1999, Friedman A, 1996, Woolley, 1990, Alfarez, 2008).

Dalteparin, a low molecular weight heparin, possess anti-inflammatory activity. Some studies indicate that heparin and its derivates have anti-inflammatory properties and decrease several component of the inflammatory process, including IL-1 and IL-6 (E. Elsayed, 2003, Jansen PGM, 1995). Previous studies have shown that both UFH (Ultra Fractionated Heparin) and LMWH (Low Molecular Weight Heparin) protect neurons and improve neurological impairments against brain ischemia (Mary, 2001; Ryu, 1996). The purpose of this study was to investigate the effect of dalteparin hippocampal neuronal death in chronically stressed rats.

2. Methods

2.1. Animals

60 adult male wistar rats, weighing 200-250 g., were used in this experiment. Animals were housed three per cage in standard condition with 12 hr light/dark cycle, 20-22°C; 45-55% humidity, free access to food and water and with lights on at 6:00 AM. The experiments were according to the rules of ethical committee for animal experiments in Tehran University of Medical Sciences. All efforts were made to minimize the animal pain or discomfort. Seven days after adaptation, the rats were randomly divided into three groups: control, stress (12 animals/group) and stress + dalteparin (12 animals/dose of dalteparin). Animals in stress and stress+dalteparin groups were exposed to Chronic Mild Stress (CMS) for 4 consecutive weeks. The rats in the stress + dalteparin group (SD) received dalteparin (Pharmacia) 70,100 and 140 IU/kg/days i.p. during stress.

2.2. Establishment of Chronic Mild Stress Models in Rats

The CMS procedure which was used in this study was adapted from the procedure described by Janne Gronli and collaborators with modifications (Janne Grønli RM, 2005). Animals were exposed to the following stressors in random order: paired caging (3hr), water deprivation (18hr) immediately followed by 1hr exposure to an empty bottle, food deprivation (18hr) immediately followed by 1h of restricted access to food (5micropellets), wet cage (300 ml water in 100g sawdust bedding) 21hr, 45° cage tilt, continuous light (36hr) and forced swimming (10min). Control animals were left undisturbed in home cages with the exception of general handling.

2.3. Blood Sampling

Following CMS period, animals were anesthetized with chloral hydrate (400mg/kg) and sacrificed via decapitation between 10:00 am and 12:00 pm, and blood (5ml) was collected in chilled tubes. The samples were centrifuged at 4 °C at 4000 rpm. The separated serums were stored at -20°C until assayed for circulating IL-6. The concentration of IL-6 in serum was measured using Enzyme-Linked Immunosorbent Assay (ELISA) kits (eBioscience) according to manufacturer's instructions.

2.4. Nissl Staining Procedure

24hr after the last day of stress exposure the rats were anesthetized with ethyl ether and perfused intracardially with PBS followed by 4% paraformaldehyde. The brains were then removed and post-fixated 48hr in the same solution, and then were processed for histological analysis by Nissl staining. Coronal paraffin sections, 7μm thick at the level of – 3.80 mm to – 4.1 mm bregma (Paxinos and Watson, 1986), were stained according to the Nissl method. Two continuous fields in hippocampal CA1, CA3 and hilus subfields were selected for each section. Cells were counted using an optic microscope at final magnification 400×, with LYSIA Bioreport software.

2.5. Statistical Analysis

All data were presented as the Mean and S.E.M. of the ratio. The number of neurons, and the serum level of IL-6 was analyzed statistically by one-way ANOVA with SPSS 16.0. Followed by post-hoc Tukey's test for multiple comparisons; Statistical significance was regarded as $p < 0.05$.

3. Results

3.1. Serum IL-6

Our results show that the serum concentration of IL-6 were significantly higher in the CMS group than in the control group ($p < 0.05$, Fig.1). We also found that dalteparin can reduce serum IL-6 level in Stress + dalteparin group in a dose dependent manner.
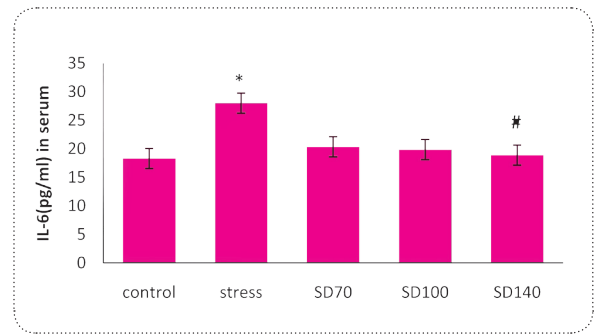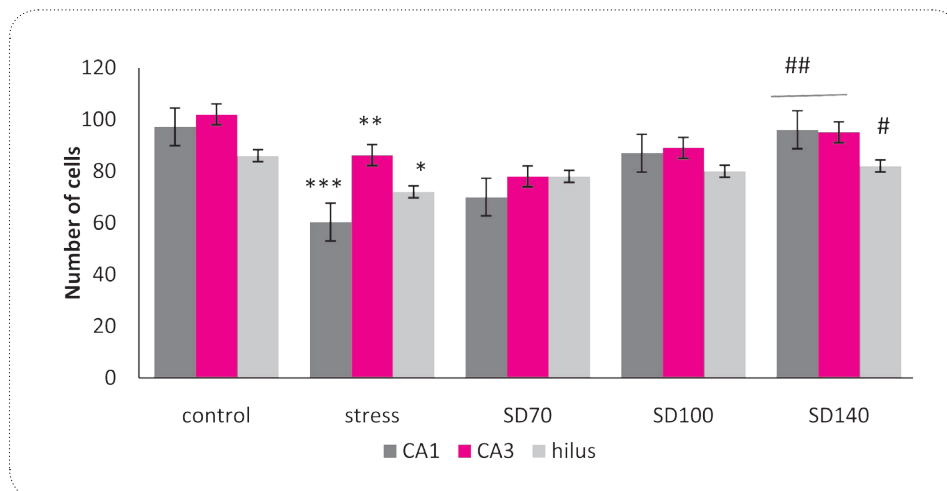

Figure 1. concentration of IL-6 in serum (Mean± SEM., n=8,* $p < 0.05$ vs. control group #, $p < 0.05$ vs. stress group)c

3.2. Assay of Number of Hippocampal CA1, CA3 and Hilus Neurons

Chronic stress resulted in significant cell loss of hippocampal CA1 (from 97.18±4.01 to 60.28±4.07, $p < 0.001$), CA3 (from 102±4.20 to 86.23 ± 3.40, $p < 0.01$) and hilus (from 86±2.3 to 72.03±1.8, $p < 0.05$) neurons, as compared to the control group. Dalteparin markedly inhibited the decreases in number of hippocampal CA1, CA3 ($p < 0.01$) and hilus ($p < 0.05$, Fig.2) neurons caused by chronic stress.

4. Discussion

The present study investigated the influence of dalteparin on hippocampal neurons during chronic stress. Prolonged stress is able to induce neuronal damage in the brain. Moreover, there is evidence that stress leads to oxidative injury in brain. Chronic stress leads to the

Figure 2. CA1, CA3, hilus neuronal cell count (Mean± SEM., n=10), *** P<0.001(CA1 subfields), ** p<0.01(CA3 subfields),*p<0.05(hilus subfields) vs. control, ## P<0.01 vs. stress group (CA1 and CA3), #p<0.05 vs.stress group (for hilus).
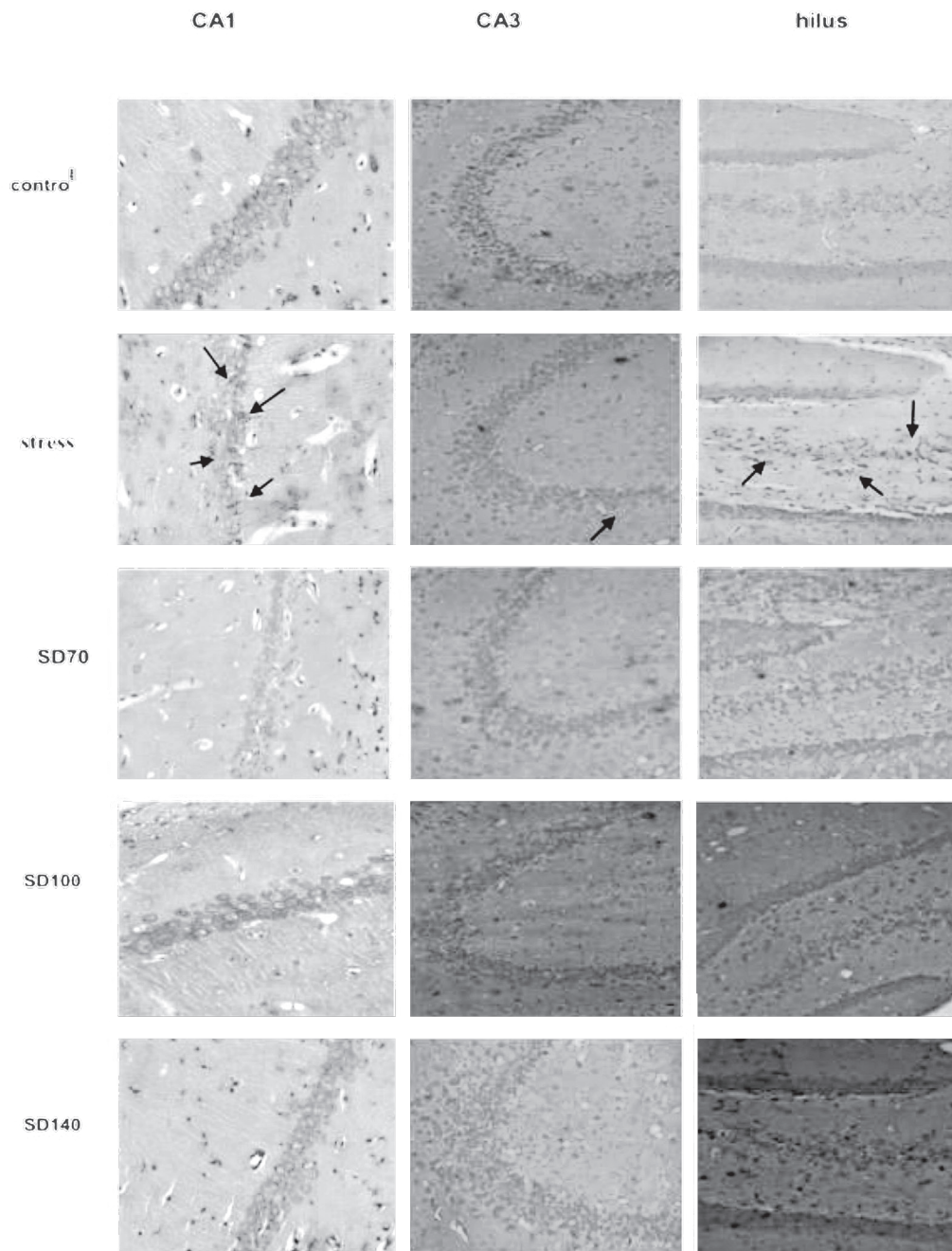
Figure 3. Nissl staining of hippocampal CA1 (Final magnification 400X), CA3 and hilus (Final Magnification 200 X) regions. Chronic stress resulted in significant neuronal cell damage (arrays indicated neuronal death) and decreased number of survival cells in the CA1, CA3 and hilus. Dalteparin reduced the ratio of neuronal death observed in hippocampus in CA1, CA3 and hilus regions.

development of a number of neural changes that may precipitate the onset of psychiatric disorders (Kendler KS, 1997, Caspi A, 2003). It is therefore important to identify the causal mechanisms that mediate the stress response in order to develop effective clinical intervention strategies. Our results confirmed the results from other laboratories that chronic stress induces loss of hippocampal neurons (Hu WP, 1998, Magarinos AM, 1995). The present study demonstrated that dalteparin reduces the ratio of neuronal death observed in hippocampus in CA1, CA3 and hilus regions by chronic stress in rats, thus suggesting that it possess a neuroprotective

effect in stress condition. Previous evidence suggests that there is a correlation between stress and inflammation, perhaps involving neurohormonal and immune mechanism (Maes, 2001, Maes 1999, Grippo AJ, 2005). Sensitivity of neurons to inflammation depends on both the duration and region affected. We suggest that inflammation induced by chronic stress leads to neuronal damage and loss of neurons in hippocampus. In accordance with the literature, our findings also demonstrated that IL-6 increases in CMS (Xiu LJ, 2010). Moreover our results showed that dalteparin inhibits the increase of serum IL-6 during stress and has its best effect at 140 IU/kg. Our results, actually demonstrated that dalteparin, when injected i.p. during stress, inhibits the neuronal damage in the hippocampus. This study shows that administration of dalteparin before stress inhibits loss of hippocampal neurons. It indicates that dalteparin could protect hippocampal neurons from damage induced by chronic stress.

Dalteparin significantly reduced the effect of CMS; supporting the hypothesis that anti-inflammation effects could oppose the stress-induced loss of neuronal network by increasing neuronal survival. We found that treatment with dalteparin during stress produces significant protection and reduces neuronal loss of hippocampus. This neuroprotective effect is manifested by preventing cell loss in the hippocampus through reduced inflammatory cytokines such as IL-6 and oxidative stress. All of these data allow us to suggest that inflammation initiated by chronic stress leads to increased damage and loss of neurons. However, damage produced by stress can be prevented, as dalteparin preserves cell survival by inhibiting the rise of IL-6 levels during stress.

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