

# Differential effects of sleep loss and chronic stressors on lipid metabolism

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## Abstract

**Background and objectives:** Stress has been implicated in the pathogenesis of several diseases. In this study, we evaluated the effects of different chronic modalities of stress, namely paradoxical sleep deprivation (PSD), electrical footshock (FS), forced swim (SW), and restraint (R), on blood parameters associated with cardiovascular risk in adult male rats.

**Methods:** FS and SW were applied twice per day for periods of one hour at 09:00 and 16:00h. Restrained animals were maintained in plastic cylinders for 22 h/day, whereas PSD was continuous. Rats were submitted to all stress modalities for four consecutive days.

**Results:** Our results demonstrated that PSD induced a significant increase in high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol concentrations and led to a reduction in the levels of triglycerides and very low-density lipoprotein (VLDL) cholesterol. SW and R groups showed a decrease in total cholesterol and HDL cholesterol fractions, and FS increased LDL cholesterol concentrations. The PSD group displayed the highest concentrations of HDL and LDL compared to other groups (SW and R).

**Conclusion:** These results suggest that each type of stress produces distinct effects on lipid metabolism.

**Keywords:** Sleep deprivation, stress, cholesterol, triglycerides, LDL, corticosterone, cardiovascular risk.

## Introduction

The physical and emotional challenges encountered during modern life have led to an increase in the prevalence of stress in the global population. Furthermore, the role of stressful life events in the etiology of several diseases has been well documented. Chronic stress in animals or humans causes endocrine alterations (1,2), memory and concentration deficits (3-6), and disturbed sleep (7,8), among other effects. The hypothalamic-pituitary-adrenal (HPA) axis is important in maintaining basal and stress-related homeostasis in the nervous system, as well as preserving cardiovascular, immune and metabolic functions (9).

Changes in the levels of particular lipids and lipoproteins in response to stress have been recognized for a long time, but the patterns and types of stressors causing these changes have not been completely consistent. Understanding these relationships is particularly important for elucidating the mechanisms responsible for stress-induced lipid alterations (10,11) and for determining a method to reduce the comorbidities involved in cardiovascular diseases. Indeed, it has been stated that heightened cardiovascular reactivity to stress contributes to the development of future cardiovascular disease (12). Furthermore, Ekstedt et al. (13) reported that sleep fragmentation is associated with elevated levels of metabolic and cardiovascular risk indicators of stress-related disorders.

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Recently, we demonstrated that paradoxical sleep deprivation (PSD) for four days has significant effects on lipid metabolism, such as increasing low-density lipoproteins (LDL) (14) and hormonal levels in male rats. When different chronic stressors were applied, PSD and footshock led to lower testosterone levels, while progesterone and corticosterone concentrations were elevated (1). In addition, cholesterol concentration was significantly decreased in rats exposed to cold environmental temperatures, indicating increased corticosterone synthesis and activation of the HPA system (9). These results indicate that the pattern of physiological responses to stress is dependent upon the stimulus applied. Thus, it may be predicted that different stressors lead to distinct changes in lipids and lipoproteins in animal models.

Collectively, these findings suggest that elevations in lipids and lipoproteins during stress are biologically and potentially clinically meaningful (10,11). However, there have only been a few studies about the effects of stressors on biochemical markers like cholesterol and triglycerides, which are related to cardiovascular risk. Thus, the aim of this study was to examine the influence of chronic stress (sleep deprivation, restraint, electrical footshock, and forced swim) on blood parameters associated with cardiovascular risk in adult rats.

## Material and Methods

### Subjects

Fifty-two male Wistar rats bred in our facilities, thirty months of age, were used in this experiment. Before applying the stressors, the rats were allowed to move freely in cages under conditions of alternating light (7:00 to 19:00h) and darkness (19:00 to 7:00h) at a room temperature of  $22 \pm 1^\circ\text{C}$ . Laboratory chow and water were provided ad libitum. All procedures used in the present study complied with the Guide for the Care and Use of Laboratory Animals, and the experimental protocol was approved by the Ethics Committee of UNIFESP (CEP #N064/99).

### Experimental procedure

The animals were assigned randomly to stress or control groups of 10-12 rats each. The control group consisted of animals that remained inside their home cages and were not manipulated except for routine cage cleaning. The experimental animals were submitted to one modality of chronic stress applied repeatedly for four days as described below and according to previous studies by Tufik et al. (15) and Papale et al. (7).

**Forced swim.** The rats were introduced individually to a 23-cm-high container filled with water at  $22\text{--}24^\circ\text{C}$ . The rats swam twice a day for a 1 h session. After the session, they were removed, allowed to dry and returned to their home cages. The experiment was carried out at 9:00 and 16:00 h.

**Restraint.** The animals were maintained in plastic cylinders (21 cm in length x 6 cm in diameter) for 22 h/day. Twice per day for 1-h periods at 9:00 and 16:00 h, the rats were allowed to move freely in their cages to eat and drink.

**Footshock.** The animals were placed individually in the compartments (14 x 25 x 28 cm) of an acrylic box containing an

electrified grid on the floor, through which the shocks were delivered. The shock intensity was 2 mA, lasting 0.25 s at intervals of approximately 15 s. Four to six shocks were delivered per minute with a variable inter-shock interval, which was changed every 5 min in order to prevent anticipation by the animal. The shocks were applied twice per day for periods of 1 h at 9:00 and 16:00 h.

**Paradoxical sleep deprivation.** The PSD method consisted of placing 10 rats in a tiled water tank (123 x 44 x 44 cm) for 96 h. The tank contained 14 platforms (6.5 cm in diameter) immersed in water up to 1 cm from their upper surface. The rats could thus move around inside the tank by jumping from one platform to another. When they reached the paradoxical phase of sleep, muscle atonia set in, and they fell into the water and awoke. Control rats lodged in the home cage showed normal sleep patterns. Food and water were provided ad libitum, and the water in the tank was changed daily. Throughout the study, the experimental room was kept at a controlled temperature ( $22 \pm 1^\circ\text{C}$ ) and with a 12:12 h light:dark cycle (lights on at 7:00 h).

### Blood sampling and biochemical measurements

All rats (control and stressed) were sacrificed immediately after the last morning application of stress at the end of the four-day period. They were sacrificed by decapitation at the same time (between 9:00 and 11:00h) with a minimum of disturbance in an adjacent room. Blood samples were collected and centrifuged at 3018 g for 10 min, and an aliquot of serum was frozen at  $-20^\circ\text{C}$ . Total cholesterol, cholesterol fractions and triglyceride concentrations were measured using a colorimetric method (ADVIA 16/50, BAYER Diagnostics Corporation). Plasma corticosterone concentrations were assayed by a double antibody RIA method designed specifically for rats and mice using a commercial kit (ICN Biomedicals, Costa Mesa, CA). The assay sensitivity was  $0.25 \mu\text{g/dL}$ .

### Statistical analyses

Values shown are expressed as mean  $\pm$  standard error of mean (SEM). Homogeneity of variance was assessed by the Bartlett test and normal distribution of the data by the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) was used to analyze the blood parameters. The Dunnett post-hoc test was used to compare values at different time points. When the Bartlett test showed an absence of homoscedasticity (unequal variances), the data were analyzed by Kruskal-Wallis ANOVA followed by the Mann-Whitney U Test.  $p < 0.05$  was considered statistically significant.

## Results

The total cholesterol (Fig. 1A) levels were significantly different in the five groups studied [ANOVA:  $F_{(4,41)} = 8.67$ ;  $p < 0.001$ ]. The total cholesterol levels were reduced in the SW and R groups compared to the control group ( $p < 0.02$  and  $p < 0.001$ , respectively). The serum triglyceride concentrations were reduced after four days of PSD [Kruskal-Wallis test:  $H_{(4,52)} = 24.9$ ;  $p = 0.001$ ] compared to the control, FS, SW and R groups ( $p < 0.001$ ).

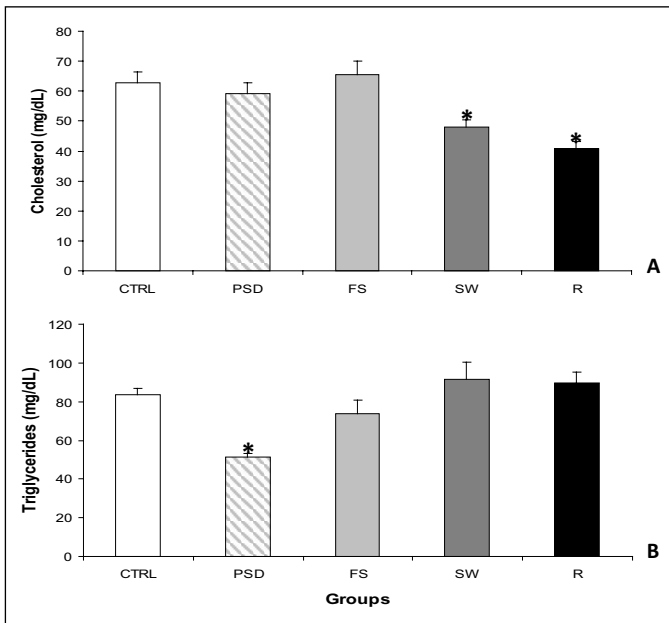


Figure 1. Total cholesterol (panel A) and triglycerides (panel B) in paradoxical sleep deprivation (PSD), footshock (FS), forced swim (SW), restrained (R) and non-stressed (control-CTRL) rats ( $n=10-12/\text{group}$ ). The values are presented as the mean  $\pm$  SEM. \*different from CTRL; ANOVA followed by the Dunnett test (total cholesterol) and Kruskal-Wallis ANOVA followed by the Mann-Whitney U Test (triglycerides).

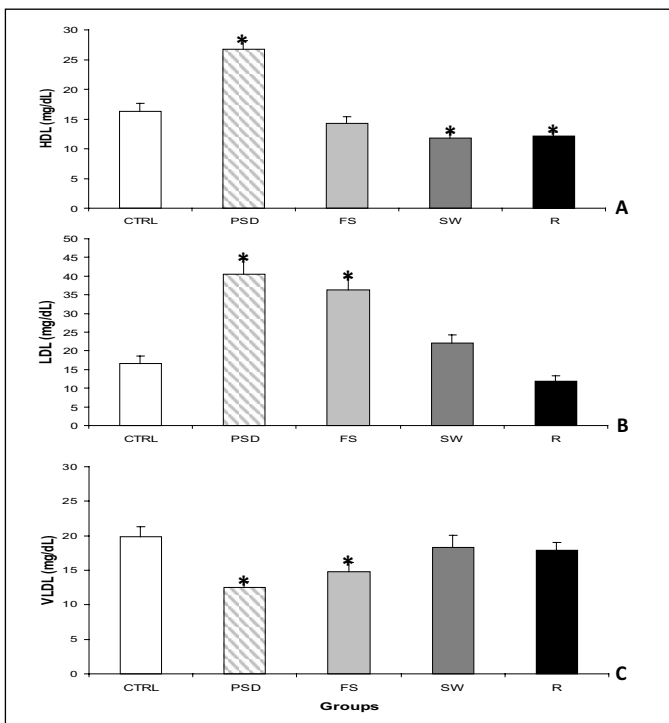


Figure 2. Cholesterol fractions of HDL (panel A), LDL (panel B) and VLDL (panel C) in paradoxical sleep deprivation (PSD), footshock (FS), forced swim (SW), restrained (R) and non-stressed (control-CTRL) rats ( $n=10-12/\text{group}$ ). \*different from CTRL; ANOVA followed by the Dunnett test.

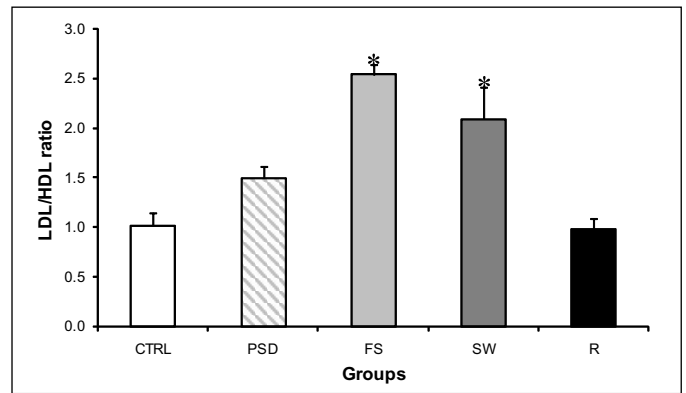


Figure 3. LDL/HDL ratio in paradoxical sleep deprivation (PSD), footshock (FS), forced swim (SW), restrained (R) and non-stressed (control-CTRL) rats ( $n=10-12/\text{group}$ ). \*different from CTRL; ANOVA followed by the Dunnett test.

The cholesterol fractions for the four groups are shown in Figure 2. HDL cholesterol [ANOVA:  $F_{(4,41)}=26.19$ ;  $p<0.001$ ; Fig. 2A] was significantly higher in the PSD group than in the control group ( $p<0.001$ ), whereas the levels in SW and R were lower than the control group ( $p<0.05$ ). The LDL fraction [ANOVA:  $F_{(4,47)}=20.41$ ;  $p<0.001$ ; Fig. 2B] was significantly increased in PSD and FS rats compared to the controls ( $p<0.001$ ).

The VLDL cholesterol values were statistically reduced by the PSD and FS protocols, as compared to the control group ( $p<0.001$  and  $p<0.04$ , respectively) [ANOVA:  $F_{(4,47)}=4.83$ ;  $p<0.01$ ; Fig. 2C]. When the LDL/HDL ratio was analyzed (ANOVA:  $F_{(4,47)}=12.02$ ;  $p<0.001$ ), the Dunnett test showed a significant difference between the rats exposed to SW ( $p<0.01$ ) and FS ( $p<0.001$ ) stress procedures compared to the control group (Figure 3).

## Discussion

The stressors applied during a four-day period were sufficient to produce marked alterations in lipid metabolism. While PSD led to a significant increase of HDL and LDL levels, triglycerides and VLDL cholesterol were decreased. The SW and R groups showed similar alterations in lipid metabolism, and both showed decreased total cholesterol and HDL cholesterol fractions. FS had no effect on total cholesterol or triglyceride levels, while LDL and VLDL were increased.

During the past two decades, considerable evidence has accumulated showing an association between cardiovascular disease (especially adverse coronary artery disease) and markers of stress and other psychological factors, such as anxiety, depression, and somatization (16-18). However, only a limited number of studies have investigated the effects of chronic stressors on biochemical blood parameters associated with cardiovascular risk in animal models. Our study is strengthened by the inclusion of sleep deprivation as a stressor, a condition not normally investigated in the majority of studies on stress-induced alterations.

Recent studies have shown that disturbed sleep and sleep deprivation are associated with subsequent occurrences of cardiovascular alterations (13,14,19-21), behavioral changes (22-24),

endocrine alterations (2,25) and overall outcomes (26,27). Chronic sleep curtailment is a consequence of voluntary bedtime restriction and is an endemic condition that affects millions of people (28,29). Sleep loss involves some degree of stress and leads to several alterations in humans (26,30) and in rodents (21-24,31-34). The platform technique used to produce PSD in the present experiment is known to be a stressful procedure (1). In fact, sleep deprivation has been associated with alterations in the regulation of the HPA axis, which can lead to harmful physiological consequences in experimental animals (35,36). For instance, several studies using the same methodology have consistently shown that PSD elicited the highest release of corticosterone (1,23,25) compared to other stress modalities. Therefore, we believe that the current results cannot be primarily attributed to the highest corticosterone levels observed after the sleep deprivation protocol. The glucocorticoid levels in these rats have been published previously (1).

Sleep deprivation was the only condition that increased HDL to a range that differed statistically from the increase due to other kinds of stressors. HDL plays an important role in cholesterol homeostasis and is strongly inversely associated with cardiovascular disease (37). HDL is thought to have a protective role, and the anti-atherogenic properties of HDL include promotion of cellular cholesterol efflux and reverse cholesterol transport, in addition to its antioxidant, anti-inflammatory and anticoagulant properties (38). However, recent data suggest that HDL does not always have an anti-atherogenic action. During the acute phase response, compositional changes occur in HDL, resulting in a loss of many of its anti-atherogenic properties. HDL may even become pro-oxidant and pro-inflammatory (39,40). Indeed, the LDL/HDL ratio represents a better assessment of cardiovascular disease risk than HDL cholesterol levels alone (41). However, according to our findings, PSD did not alter the LDL/HDL ratio, whereas the LDL/HDL ratio significantly increased after application of the FS and SW stressors. It seems that the cumulative rate of sleep deprivation, which is thought to be an aggravating factor of cardiovascular disease (42), may be due to the acute and chronic effects on cardiovascular physiology arising from changes in the autonomic nervous system (43), rather than through alterations in the cholesterol profile. Due to a scarcity of studies regarding chronic stress contexts and cholesterol ratio, the mechanism underlying this interaction is not well understood. Imbalances in the cardiovascular system which lead to an increased propensity for disease require further investigation.

Sleep loss induced an increase in LDL concentrations. These data reinforce our previous findings on the effects of PSD on lipid blood parameters (14) and could indicate that sleep deprivation may protect the cardiovascular system. Consistent with the literature (14,21), our findings demonstrated that rats submitted to 96 h of PSD showed a reduction in triglyceride concentration. The current study, as well as previous sleep deprivation studies in rats, documents decreases in body weight (43) and hyperphagia (44) (data not shown). In fact, four days of PSD reduced body weight by 5.7% in male rats (21,43). Using the same methodology, our group has demonstrated that hyperphagia did not always occur with shorter periods of PSD (43). One might therefore hypothesize that the differences observed in triglyceride levels in PSD occurred

as a result of weight loss; however, unlike the decrease in LDL, the reduction of triglyceride concentration is not related to this weight reduction (21). Although it appears that sleep deprivation leads to an improvement in the cardiovascular system, community-based prospective studies have shown that habitual sleep duration averaging <5 h per night is associated with an increased prevalence of arterial hypertension (45-47). Studies have proposed that activation of the sympathetic nervous system by sleep deprivation may be involved in triggering cardiovascular events in the morning hours (48-50). Extended hours of wakefulness may be directly detrimental to the cardiovascular system, contributing to increased cardiovascular risk (50).

Many types of aversive stimuli have been applied to animal models on a short or long-term basis to investigate the response of the HPA axis to stress (1,51,52). Responses to stressors are dependent upon the stress stimulus applied (1,7,53-55). Rats submitted to SW and R showed lower cholesterol levels and HDL levels compared to the control group. SW and R treatment both led to similar patterns in which cholesterol and HDL cholesterol were decreased, as compared to the PSD and FS protocols. Moreover, R and SW did not lead to modifications of LDL cholesterol concentration. Apparently, R and SW are opposite stress modalities; however, both produce physical and inescapable psychological stress (56).

Among the different stressors the animals were subjected to, only the FS protocol produced changes that were not significant, as compared to the controls. Indeed, FS did not promote significant alterations in triglyceride, total cholesterol or HDL cholesterol fractions. Only LDL and VLDL were increased after the experimental period (Figure 2B). However, an increase in the LDL/HDL ratio was observed in this group. Under the same protocol, corticosterone release was significantly enhanced in the FS group compared with control and SW groups (1). Other stressors, such as SW and R, did not lead to significant alterations. It appears that stress inflicted by FS was sufficient to activate the Hypothalamic-pituitary-adrenal (HPA) axis and cause an increase in the LDL and HDL/LDL ratio. In this sense, stress-induced activation of the HPA axis may be responsible for the increase in LDL and for many of the detrimental effects inflicted by prolonged exposure to stress. Specifically, the secretion of hormones due to stress may lead to long-lasting increases in LDL, which represents a serious increase in the risk of cardiovascular disease. Although the implications of stress-induced metabolic changes are discussed, it should be noted that this is a complex scenario, in which natural differences in the response to stressful situations might be related to a variety of mechanisms, with distinct consequences of different types of stress and different interactions with the HPA axis. Although activation of the HPA axis is the principal effect of most stressful situations, it is not the sole cause of the results, and well-balanced coordination among multiple steroid hormones besides cholesterol might be expected after stress. Papale et al. (7) reported that each chronic stressor promotes different changes. For instance, R was the only stressor that resulted in a significant decrease in sleep throughout the entire period of recording. SW reduced slow wave and augmented paradoxical sleep only on the first day of stress exposure. FS only initially produced alterations in sleep efficiency and

decreased slow wave sleep/paradoxical sleep. Collectively, the discrepancies between sleep architecture and lipid metabolism show that the pattern of change depends on the nature of the stressful experience and the resultant coping strategies. Additional studies are warranted to investigate the complex relationship between cholesterol, other cardiovascular parameters, and the magnitude of sleep loss in chronic stress-related conditions.

In conclusion, the response of the cardiovascular system to stress seems to be stressor-specific, and the pattern of the physiological response determines how a given organism copes with stress. While chronic stress may induce an altered endocrine scenario, other conditions may lead to prompt action of the HPA axis in an effort to normalize the response to stress. Although it is methodologically difficult to design two or more conditions with equivalent stress intensities and parallel effects on cardiovascular variables, further studies are warranted to examine other parameters that may be more informative markers of cardiovascular risk.

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