

## **Homocysteine levels, oxidative status and hemostatic response to chronic stress in rats**

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### **ABSTRACT**

Several studies have shown that homocysteine (Hcy) levels are affected by diet factors and non-diet factors such as stress. Increases in total Hcy plasma concentrations are recognized as an independent risk factor for cardiovascular disease. It has been demonstrated that elevated circulating levels of Hcy cause changes in hemostasis by altering the vascular endothelium function, changing the character of its surface from anticoagulant to pro-coagulant. On the other hand, it has been demonstrated that psychosocial stress increase the plasmatic levels of pro-thrombotic factors. The aim of this study was to evaluate the Hcy levels and oxidative status in the haemostatic response to chronic immobilization stress in rats. Two groups of Male Wistar rats were considered: control group (C) and immobilization stress group (IMO). The rats were stressed for 2 h (from 10.00 AM to 12.00 PM) for 14 days. On day 14 immediately after the last IMO, the animals were killed by decapitation. Blood samples were taken and Clotting time (CT), partial thromboplastin time (APTT), platelet count, fibrinogen, Hcy, thiobarbituric acid reactive substances (TBARs), antioxidant capacity of plasma (FRAP), total nitrites (NO<sub>x</sub>), and corticosterone levels were determined. Correlation test between Hcy and CT, NO<sub>x</sub> and Corticosterone were made. An increase in plasma corticosterone, platelet count, and fibrinogen and homocysteine levels in response to stress was observed. FRAP, CT, APTT, and NO<sub>x</sub> serum values were lower in stressed animals while no significant differences in TBARs plasma concentration to IMO were found. Positive correlation was found between Hcy and corticosterone levels and negative correlation for Hcy and NO<sub>x</sub> and Hcy and CT was observed. IMO chronic stress modifies the hemostatic response leading to a pro-thrombotic state with an increase of platelets, fibrinogen and Hcy. The rise in Hcy appears to depend on corticosterone levels. Increased Hcy decrease NO bioavailability, which would promote platelet adhesion to endothelial cells favoring the formation of blood clots in response to chronic stress situations, which would increase the risk of thromboembolic events in situations of chronic stress.

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### **KEYWORDS**

Chronic stress;  
Hemostasis;  
Thrombosis;  
Cardiovascular risk;  
Homocysteine;  
Oxidative status.

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### INTRODUCTION

Homocysteine (Hcy) is a sulphur-containing amino acid that is an intermediary in the metabolism of methionine–cysteine. This metabolic pathway is important due to the production of S-adenosylmethionine, the main donor of methyl groups for methylation reactions in the organism. Hcy can be converted into methionine via a re-methylation pathway that requires folate and vitamin B12, and/or into cysteine via a trans-sulfuration pathway that requires vitamin B6. Hcy can also be exported to the extracellular environment<sup>[1]</sup>.

Several studies have shown that levels of Hcy are affected by diet factors and non-diet factors such as stress<sup>[2]</sup>. Increases in total Hcy plasma concentrations are recognized as an independent risk factor for cardiovascular disease<sup>[1]</sup>. It has been demonstrated that elevated circulating levels of Hcy cause changes in hemostasis by altering the vascular endothelium function, changing the character of its surface from anticoagulant to pro-coagulant<sup>[3,4]</sup>. Moreover, increased Hcy causes decreased bioavailability of nitric oxide (NO), increased oxidative stress and low-density lipoprotein oxidation, platelet activation and inhibition of tissue plasminogen activator (t-PA)<sup>[5]</sup> although the mechanisms have not been fully elucidated.

As above mentioned, Hcy levels can be altered by some stressors. De Souza<sup>[1]</sup> have previously reported that when female Wistar rats were subjected to various acute stressors, only restraint stress resulted in increased Hcy concentrations. Moreover, few studies have attempted to correlate chronic stress and Hcy levels.

On the other hand, von Kanel<sup>[6]</sup> has shown that psychosocial stress increased the plasmatic levels of prothrombotic factors. Sympathetic activation appears to be involved in modulation of fibrinolytic activity and antithrombin III levels. Chronic stress stimulate  $\beta$ 1-adrenergic receptors on the vascular endothelium, this would lead to reduced intracellular synthesis of prostacyclin which impairs release of t-PA and also it causes the inhibition of circulating plasminogen activator inhibitor-1 (PAI-1)<sup>[7]</sup>.

Moreover, although there is no direct relationship between hypercoagulability and oxidative

stress, it is known that endothelial activation by reactive oxygen species (ROS) or their final products induce the expression of adhesion molecules resulting in increased cell adhesion and platelet activation. Induced endothelial changes by oxidative stress enhance the intraluminal exposure of tissue factor, which initiates the coagulation cascade through binding with factor VII. By reducing NO bioavailability, oxidative stress inhibits the antiadhesive and antithrombotic properties of NO further enhancing the pro-coagulant state<sup>[8]</sup>.

Taking into account that there is few works that to investigate type and duration of the stress exposure on the Hcy levels and that Hcy would have a pro-thrombotic and oxidative action, the aim of this study was to evaluate the Hcy levels and oxidative status in the haemostatic response to chronic immobilization stress in rats.

### METHODS

#### Animals and general conditions

Male Wistar rats (n=16) weighting 250-300 g, were maintained under standard conditions (kept with the light on from 07.00 AM to 07.00 PM, at  $20 \pm 2$  °C) in individual cages, with wood shaving bedding with food and water *ad libitum*. Two groups of eight rats each were considered: control group (C) and immobilization stress group (IMO). The rats were stressed for 2 h (from 10.00 AM to 12.00 PM) by taping their four limbs to metal mounts attached to wooden boards as it was previously described by Receptekova and Mikulaj<sup>[9]</sup> for 14 days.

#### Samples

On day 14 immediately after the last IMO, the animals of the two groups were killed by decapitation. Blood samples were taken and clotting time (CT) and platelet count was determined. Other aliquots were centrifuged for 10 min at 3,000 g, and partial thromboplastin time (APTT), fibrinogen, homocysteine, thiobarbituric acid reactive substances (TBARs), antioxidant capacity of plasma (FRAP), total nitrites (NOx), and corticosterone levels were determined.

#### Determinations

Clotting time was determined by Lee-White

method and platelet counts were made in a hematology analyzer. Plasma corticosterone levels were measured by radioimmunoassay (RIA) as described previously by Armario and Castellanos<sup>[10]</sup> with a modification: corticosterone-binding-globulin was denatured by heating the samples at 70°C for 30 min. Inter and intra-assay coefficients of variation were 15 and 10 per cent, respectively.

Plasma thiobarbituric acid reactive substances (TBARs) concentrations, expressed as nmol MDA/g of tissues were measured spectrophotometrically at 532 nm by the method of Okawa et al<sup>[11]</sup>

The total homocysteine (free and protein-bound homocysteine) in plasma was measured as described by Minniti et al.<sup>[12]</sup> The homocysteine derivatives are separated by reversed-phase high-performance liquid chromatography (HPLC) followed by fluorescence detection.

Antioxidant capacity of plasma (FRAP) was determined based on the ferric reducing ability of plasma, following the technique of Benzie and Strain<sup>[13]</sup>

Activated partial thromboplastin time (APTT) and fibrinogen was determined with a commercial kit (Wiener Lab®, Argentina) and Nitric oxide (NO) was estimated through its stable metabolites NO<sub>2</sub>, NO<sub>3</sub> and Total NO<sub>2</sub> (NOx) by the Griess method<sup>[14]</sup>.

The statistical significance was evaluated by the statistical software STATISTICA (Statasoft). One-way analysis of variance (ANOVA) was performed. Correlations between Hcy and corticosterone, clot-

ting time and NOx were performed by Pearson's correlation test.

## RESULTS

ANOVA revealed an increase in plasma corticosterone and homocysteine levels in response to stress ( $p=0.0002$  and  $p=0.0002$  respectively). Antioxidant capacity of plasma and NOx serum values were significantly lower in stressed animals ( $p=0.008$  and  $p=0.00003$  respectively) while no significant differences in TBARs plasma concentration in response to IMO were found (TABLE 1).

Higher plasma fibrinogen levels and platelet count were found ( $p=0.0002$  and  $p=0.009$ , respectively) while short clotting time and plasma APTT were observed in stressed animals ( $p=0.01$  and  $p=0.03$ , respectively) (TABLE 2).

There was a significant positive correlation ( $r=0.7015$ ,  $p=0.006$ ) between Hcy and corticosterone (Figure 1), while a negative correlation between Hcy and CT ( $r= -0.80$ ,  $p= 0.002$  Figure 2) and Hcy and NOx ( $r = -0.85$ ,  $p= 0.002$  Figure 3) was observed.

## DISCUSSION

The haemostatic system and its main parts: the endothelium, blood platelets, blood coagulation and fibrinolysis, regulate the balance between thrombotic and antithrombotic status. An imbalance in this com-

**TABLE 1 : Plasma corticosterone, Hcy, FRAP, NOx and TBARs in response to chronic stress**

	Control	Stress
Corticosterone (µg/dL)	6.98 ± 0.83	31.08 ± 3.03 a
Hcy (µmol/L)	5.38 ± 0.23	9.37± 0.57 b
FRAP (µmol/L)	511.5 ± 38.8	322.1 ± 51.1 c
NOx (µmol/mL)	5.88 ± 0.53	1.08 ± 0.19 d
TBARs (µmol/dL)	1.4 ± 0.46	1.7± 0.58

Means ± SEM are represented. a  $p= 0.00003$ , b  $p=0.008$ , c  $p=0.0002$ , d  $p=0.0002$

**TABLE 2 : Plasma fibrinogen levels, platelet count, CT and APTT in response to chronic stress**

	Control	Stress
Fibrinogen (mg/dL)	205 ± 10	330 ± 12 <sup>a</sup>
Platelet count/mm <sup>3</sup>	480.000 ± 115.000	641.115 ± 78.000 <sup>b</sup>
CT (seg)	120.33 ± 2.04	82.67± 1.20 <sup>c</sup>
APTT (seg))	41.20 ± 2.10	37.60 ± 1.20 <sup>d</sup>

Means ± SEM are represented. a  $p=0.009$ , b  $p=0.01$ , c  $p=0.03$ , b  $p=0.0002$ .

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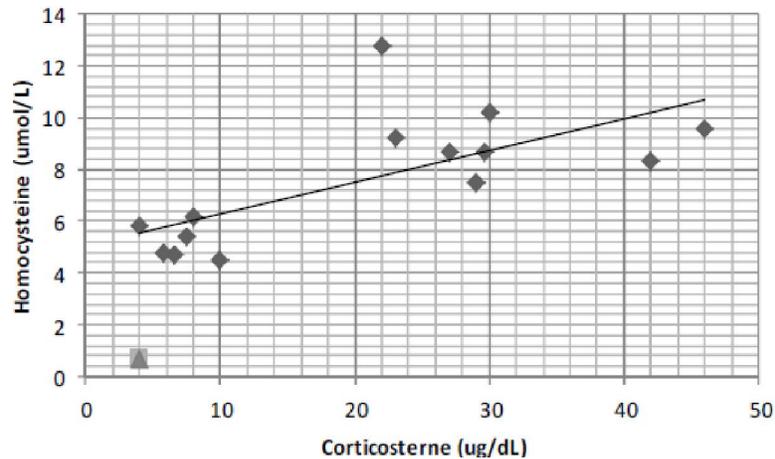


Figure 1 : Correlation between homocysteine and corticosterone.  $r=0.7015$ ,  $p=0.006$

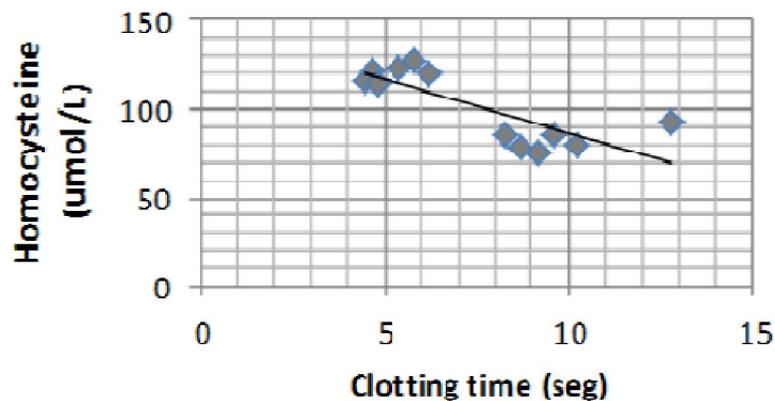


Figure 2 : Correlation between homocysteine and clotting time  $r= 0.8$ ,  $p=0.002$

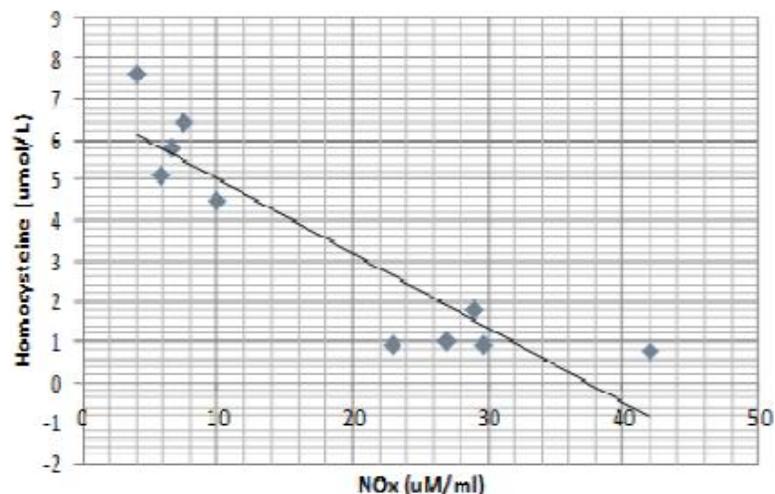


Figure 3 : Correlation between homocysteine and NOx.  $r= 0.85$ ,  $p=0.002$

plex system proceeds towards an enhanced risk of cardiovascular diseases.<sup>[15]</sup>

Increases in the clotting factors such as FVII, FVIII and FXII, thrombin–antithrombin complex, fibrinogen and fibrin levels in response to acute psychological stress has been shown, suggesting that acute psychological stress affect both the intrinsic (FXII) and extrinsic (FVII) coagulation pathways,

as well as in the final stages of the common pathway (fibrinogen)<sup>[16]</sup>.

In the present work, as it was expected, higher corticosterone levels were found in response to stress, reflecting the activation of the hypothalamic-pituitary axis. Coincidentally decreased clotting time and APTT in response to chronic immobilization stress has been observed. These results agree with

the view that stress can cause changes in haemostasis.

Moreover, increased fibrinogen levels and platelet count were found. Austin et al.<sup>[17]</sup> found an increase in fibrinogen levels and platelet count in healthy subjects with acute psychosocial stress, indicating an activation of haemostatic process. Similarly, in chronic stress situations, pro-coagulant markers, particularly fibrinogen and d-dimer, are augmented and fibrinolysis activity is diminished, which could lead the hemostatic balance between coagulation and fibrinolysis towards chronic hypercoagulability, which potentially increment the risk to thrombotic diseases<sup>[17, 18]</sup>.

In addition to increases in fibrinogen levels and platelet count, catecholamine secretion is increased during stress and could activate platelets directly through  $\alpha_2$ -adrenergic receptors<sup>[19]</sup>. This would be an additional factor that stimulates a pro-coagulant state.

Furthermore, in this work, an increase of 37% in Hcy levels in chronically stressed rats was observed. Plasma Hcy concentrations are dependent on complex metabolic regulation<sup>[20]</sup>. Some of these regulatory systems are activated by stress and could mediate the changes observed in Hcy levels in this work.

Other authors have registered hyperhomocysteinemia in response to restrain stress but not in response to other stressors such as swim, novelty and cold<sup>[20,1]</sup> indicating that Hcy changes occurred in a stressor-specific manner. Moreover, de Souza et al.<sup>[1]</sup> demonstrated that changes in Hcy concentrations in response to acute stress do not seem to be directly related to corticosterone levels. However, in our study we found a little but positive correlation among corticosterone and Hcy levels showing a dependency between both variables. It is noteworthy that in our work a chronic stress was performed while in the other works an acute stress was used. Furthermore, IMO is a strong stress and involucre physical and psychological components. In future works will be taken into account the complexity of the physiological responses to stress and regulatory systems involved in homocysteine metabolism in order to clarify the IMO effect on total plasma homocysteine concentrations.

Increased Hcy level would promote platelet ad-

hesion to endothelial cells and has also been associated with higher levels of prothrombotic factors for example,  $\beta$ -thromboglobulin, tissue plasminogen activator and factor VII, leading to the augmentation of thrombus formation<sup>[21, 22]</sup>. Furthermore, hyperhomocysteinemia is associated with the formation of fibrin with thinner and more tightly packed fibers and increased resistance to fibrinolysis<sup>[23]</sup>. Moreover, by the trans-sulfuration pathway, Hcy is transformed to hydrogen sulphide ( $H_2S$ ) which is involved in prothrombotic events<sup>[24, 25]</sup>. In this work, a negative correlation between Hcy and clotting time was found, indicating a certain participation of hyperhomocysteinemia on the shortening of the clotting time.

On the other hand, the auto oxidation of Hcy could be one of the sources of ROS production<sup>[26]</sup>. Furthermore, stress conditions have been associated with enhanced free radical generation causing oxidative stress<sup>[1]</sup>. In our work a decrease in plasma antioxidant capacity and NOx levels were found without changes in plasma TBARS levels. Probably, the plasma antioxidants are consumed for maintain TBARS without changes, reflecting an antioxidant protection. In addition, the minor plasma antioxidant capacity found in stressed animals is consistent with the highest Hcy levels. Hence, these results would support a pro-oxidant effect of high concentrations of Hcy. However, immobilization stress also could cause an increment in ROS. Therefore, the high Hcy levels and stress would be two factors that enhance oxidative stress in this work.

On the other hand, it is known that ROS are implicated in propagation of platelet activation by inactivating NO and releasing platelet agonists such as ADP<sup>[27]</sup>. However, the introduction of additional oxidative stress in certain settings may be prothrombotic. Adhesion of platelets to the endothelium is prevented by several mechanisms, including endothelial cell production of prostacyclin and NO<sup>[28]</sup>. In the present study, low NOx levels, indicative of lower NO bioavailability in stressed than control rats was found. NOx levels were negatively correlated with the Hcy indicating the relationship between high Hcy levels and NO bioavailability. This would be an additional factor that contributes to the hypercoagulability in response to chronic

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stress.

In conclusion, IMO chronic stress modifies the hemostatic response leading to a pro-thrombotic state with an increase of platelets, fibrinogen and homocysteine.

The rise in Hcy levels appear to depend on corticosterone levels. Increased Hcy level decrease NO bioavailability, which would promote platelet adhesion to endothelial cells favoring the formation of blood clots in response to chronic stress situations, which would increase the risk of thromboembolic events in situations of chronic stress.

Disclosure section: we have not any conflict of interest.

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