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RESEARCH ARTICLE

The Effects of Restraint and Cold Restraint Stress on Coagulation Indicators in Wistar Albino Rats

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ABSTRACT

The aim of this study was to investigate the effects of restraint and cold restraint stress from acute stress protocols on coagulation indicators in rats. The study was conducted in 18 male Wistar albino rats aged 8-10 weeks with a body weight of 180-220 g. After a one-week adaptation period, the rats were randomly divided into three groups (n=6/group). The animals in the control group were not exposed to any stress. Rats in the restraint group were housed in restrainers designed for rats, and their movement was restricted for 2 hours at room temperature. Rats in the cold restraint group were kept in the restrainer at +4°C for 2 hours. Blood samples were collected by cardiac puncture under ketamine and xylazine anesthesia in Vacutainer® tubes containing 3.2% sodium citrate (0.109 M trisodium citrate). Coagulation indicators (aPTT, PT, INR, fibrinogen, and D-dimer) were analyzed using an automated analyzer (Roche Cobas t511, Switzerland). Although acute stress (restraint and cold restraint stress) had no effect on aPTT and D-dimer levels ($p>0.05$), it increased PT and INR values ($p<0.05$) and decreased fibrinogen concentration ($p<0.05$). Consequently, acute stress may lead to platelet hypofunction in rats by prolonging PT, increasing INR, and decreasing fibrinogen concentration.

Keywords: Acute stress, aPTT, D-dimer, Fibrinogen, INR, PT

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Kısıtlama ve Soğuk Kısıtlama Stresinin Wistar Albino Sıçanlarda Pıhtılaşma Göstergeleri Üzerindeki Etkileri

ÖZ

Bu çalışmanın amacı, kısıtlama ve soğuk kısıtlama stresi gibi iki farklı akut stres protokolünün sıçanlarda pıhtılaşma göstergeleri üzerindeki etkilerini araştırmaktı. Çalışma, vücut ağırlığı 180-220 g olan, 8-10 haftalık 18 erkek Wistar albino sıçan üzerinde gerçekleştirildi. Bir haftalık adaptasyon sürecinden sonra sıçanlar rastgele üç gruba (n=6/grup) ayrıldı. Kontrol grubundaki hayvanlar herhangi bir strese maruz bırakılmadı. Kısıtlama grubundaki ratlar, ratlar için tasarlanmış kısıtlayıcı içinde barındırıldı ve oda sıcaklığında 2 saat hareketleri kısıtlandı. Soğuk kısıtlama grubundaki ratlar +4°C'de 2 saat kısıtlayıcı içinde tutuldu. Kan numuneleri, %3,2 sodyum sitrat (0.109 M trisodyum sitrat) içeren Vacutainer® tüplerine ketamin ve ksilazin anestezisi altında kardiyak punksiyon yoluyla toplandı. Pıhtılaşma göstergeleri (aPTT, PT, INR, fibrinojen ve D-dimer) otomatik bir analiz cihazı (Roche Cobas t511, İsviçre) kullanılarak analiz edildi. Akut stres (kısıtlama ve soğuk kısıtlama stresi) aPTT ve D-dimer düzeylerine etki etmezken ($p>0.05$), PT ve INR değerlerini artırdı ($p<0.05$), fibrinojen konsantrasyonunu düşürdü ($p<0.05$). Sonuç olarak akut stres, PT'yi uzatarak, INR'yi artırarak ve fibrinojen konsantrasyonunu azaltarak sıçanlarda trombosit hipofonksiyonuna yol açabilir.

Anahtar kelimeler: Akut stres, aPTT, D-dimer, Fibrinojen, INR, PT

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INTRODUCTION

Stress-related factors such as increased platelet activation, endothelial dysfunction, coagulation, up-regulated inflammatory response, and altered fibrinolysis are crucial in the thrombotic processes linked to cardiovascular events (Sandrini et al. 2020). Numerous research on human hemostasis indicates that acute psychophysiological stress simultaneously stimulates coagulation and fibrinolysis, but also raises coagulation further, resulting in hypercoagulability (von Känel et al. 2001, Wirtz et al. 2008). In stressful conditions, the hypothalamic-pituitary-adrenal (HPA) axis and autonomic nerve system (ANS) are activated as a part of the organism's adaptive response, releasing glucocorticoids and catecholamines (norepinephrine and adrenaline), which either directly or indirectly affect hemostasis (Sandrini et al. 2020).

Acute stress has different results on platelet reactivity in healthy subjects. Platelet surface glycoproteins, fibrinogen receptors, and P-selectin increased the expression of the organism to prevent excessive bleeding during acute stress, and the interaction with leukocytes and supports them come together (Strike et al. 2004). In addition to blood hyperviscosity, reduced plasma volume increased hematocrit, and activated coagulation factors, psychologically acute stress enhances plasma filtration in healthy individuals (Austin et al. 2012, Zgraggen et al. 2005). On the other hand, a recent study found that the expression of P-selectin on the platelet surface was significantly increased in mice after 9 hours of stress (Pethaperumal et al. 2022). It has certainly been demonstrated in different animal models that sustained and intense stress increases platelet production and activation, enhancing the ability of thrombin and ADP to stimulate platelet aggregation and promote platelet-leukocyte interaction (Matsuhisa et al. 2014, Sandrini et al. 2017). It has been documented that individuals exposed to acute mental stress tend to increase the level of D-dimer with an increase in the level of some coagulation factors (FVII: C, FVIII: C, FXII: C, von Willebrand factor antigen (vWF)) and fibrinogen (Zgraggen et al. 2005). In another study on humans, it was reported that aPTT decreased, and fibrinogen, FVIII: C, D-dimer, and PT values increased significantly in psychologically acute stress exposure (Austin et al. 2012). These inconsistencies may result from numerous study variations, including stress levels and platelet aggregation measuring techniques.

In the literature, the effects of different stress protocols (repeated cold stress, acute vital stress, immobilization stress, water immersion stress, restraint stress, cold stress) on platelet functions have been investigated in previous rat and mouse stress studies (Bondarchuk et al. 2020, Hata et al. 1992, Hatu et al. 1991, Kawabata and Hata 1993, Loban'-Chereda and Novosel'tseva 1990, Malyszko et al. 1994, Pethaperumal et al. 2022, Takeda et al. 1992).

In this study, we hypothesized that acute restraint and cold restraint stress protocols might have different effects on the coagulation profile and that mobility restriction with hypothermia might enhance the activation of the coagulation cascade. There is no experimental study on this subject in the literature. The aim of this study was to investigate the effects of restraint and cold restraint stress from acute stress protocols on coagulation indicators in rats.

MATERIAL AND METHODS

The study was carried out with approval from the Sivas Cumhuriyet University Animal Experiments Ethics Committee (Approval No: 65202830 - 050.04.04 - 640). The animals were provided by the Experimental Animals Application and Research Center of Sivas Cumhuriyet University. The animals were housed in standard care (appropriate ventilation, 12/12 h light/dark cycle, 21-23°C temperature, 35-60% humidity) and feeding (ad-libitum water and pelleted rat feed (DSA Agrifood Products Inc., Kırıkkale)) conditions. The content of pellet feed was as follows: Crude protein (24%), crude fiber (7.62%), crude oil (3%), crude ash (8.01%), phosphorus (0.75%), sodium (0.26%), and calcium (1.23%). The study was carried out on 18 male Wistar Albino rats, 8-10 weeks of age, with a body weight of 180-220 g. Rats were randomly divided into three groups (n=6/group) and underwent a one-week adaptation period to adapt to a new housing environment. Then, the rats were left quiescent for adaptation in the experimental room for 12 hours before applying the stress procedures.

Control group: Animals in this group did not be exposed to any stress. Blood samples were taken from animals simultaneously with acute stress group animals. Blood samples were taken from animals simultaneously with acute stress group animals. Blood samples were collected by cardiac puncture under ketamine (60 mg/kg, Keta-Control, Doğa İlaç, İstanbul) and xylazine (10 mg/kg, Ksilazol, Alivira, Ankara) anesthesia using 21-gauge needles, into 1.8 mL Vacutainer® tubes (Becton Dickinson, USA) with 3.2% sodium citrate (0.109 M trisodium citrate) and gently mixed six times. Blood was analyzed within 2 hours at the latest.

Restraint stress: Restraint group: In this stress protocol, rats were placed in restrictive cylinder-shaped plastics (Broome Restrainer, 63.5 x 215.9mm (2.5" dia. x 8.5") designed for the rat as previously described (Tu et al. 2019), and their movement was restricted for 2 hours at room temperature. Then, the procedure described in the control group was repeated.

Cold Restraint stress: Cold Restraint group: As previously described, rats were kept in the restrainer (Broome Restrainer, 63.5 x 215.9mm (2.5" dia. x 8.5")

at +4°C for 2 hours (Zhu et al. 2014). The procedure described in the control group was repeated.

Coagulation indicators (aPTT, PT, and fibrinogen) and D-dimer were analyzed by optical coagulometric method and latex-based immunoturbidimetric method, respectively, using an analyzer (Roche Cobas t511, Switzerland) as previously described (Kitchen et al. 2018a, Kitchen et al. 2018b). Citrated blood tubes were centrifuged at 2500 g for 15 minutes. After centrifugation, samples were checked for preanalytical errors such as correct filling of the tube and the presence of hemolytic, lipemic, or icteric plasma that could affect test results. During the study, freshly collected plasma was stored in the sealed primary tube over the cell pellet. Roche aPTT Screen Reagent contains a mixture of purified phospholipids and silica particles as an activator that stimulates the formation of factor XIIa. Calcium chloride is then added, which initiates the intrinsic coagulation cascade. Time from addition of calcium chloride to clot formation is measured. Roche PT Rec Reagent contains recombinant human thromboplastin with a heparin-neutralizing substance and calcium that, when added to citrated rat plasma, triggers activation of the extrinsic coagulation cascade. The time between addition of the reagent to plasma and formation of a fibrin clot is measured and expressed in seconds. The reagent lot-specific ISI is used to convert the patient's PT result in seconds to the international normalized ratio (INR) using the following formula INR = (patient PT / mean normal value PT) ISI. The ISI value for a given thromboplastin reagent is determined by a method comparison of the thromboplastin reagent to be standardized with an international reference thromboplastin. The fibrinogen test is a Clauss test using lyophilized bovine thrombin at a concentration of 100 NIH units/ml with added stabilizers and buffers. The D-dimer test is a particle-enhanced immunoturbidometric assay in which latex particles are coated with monoclonal antihuman D-dimer antibodies (mouse) at 0.12%. The start reagent is used together with a preservative/buffer solution at pH 8.2. Antigen/antibody complexes formed by the addition of samples containing D-dimer lead to an increase in the turbidity of the test reactants. The change in absorbance with time depends on the concentration of D-dimer epitopes in the sample. The aggregate is determined turbidimetrically.

Statistical Analysis

Data analysis was performed in GraphPad Prism 8.00 (GraphPad Software, San Diego, CA, United States). The normal distribution of the data was confirmed by the Shapiro-Wilk test. The one-way ANOVA test followed by Tukey's multiple comparisons test was used to evaluate differences in analytes. Results were expressed as mean ± SD. Values of $p < 0.05$ were considered significant.

RESULTS

PT and INR values were statistically significant and higher in the restraint and cold restraint groups compared to the control group ($p<0.05$). However, aPTT was not statistically significant between the groups ($p>0.05$). Fibrinogen concentration was statistically significant and lower in the restraint group ($p<0.001$) and cold restraint group compared to the control group ($p<0.01$) (Figure 1). Interestingly, it yielded results <0.2 in all animals in the D-dimer level groups. This value is within the reference range of 0-0.5 mg/L FEU (Table 1).

Table 1. D-Dimer concentration changes in groups.

Analyte	Groups			
	Control	**Restraint**	**Cold Restraint**	**Reference range**
D-Dimer (mg/L FEU)	<0.2	<0.2	<0.2	0-0.5

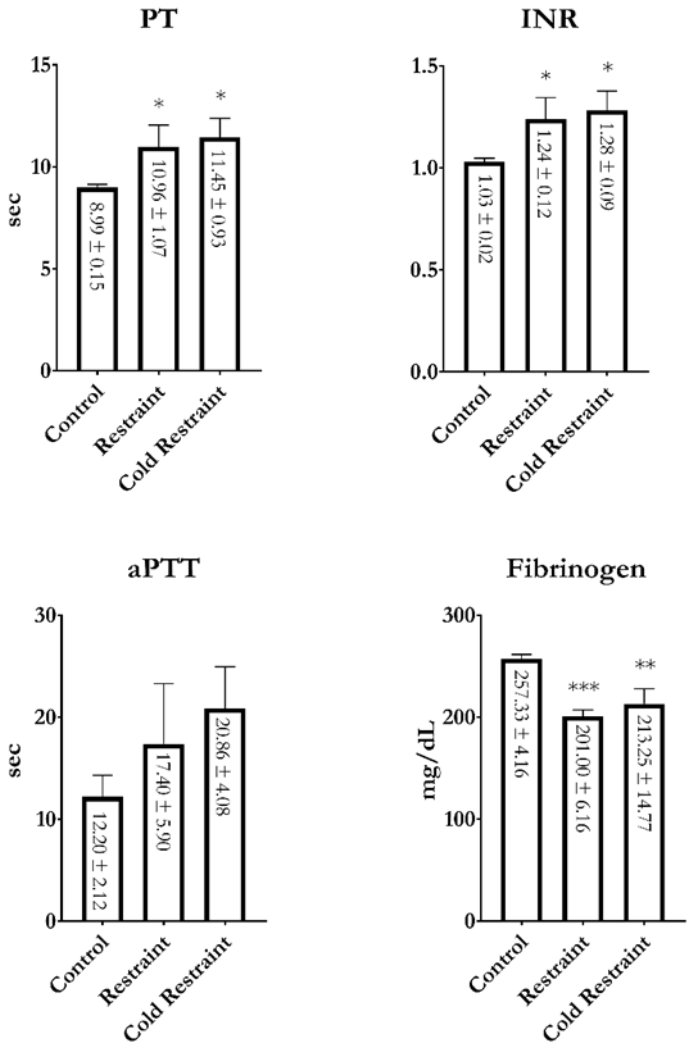

Figure 1: Coagulation parameters changes in rats exposed to restraint and cold restraint stress. *p<0.05, **p<0.01, and ***p<0.001 are statistically significant compared with control group according to one way ANOVA post hoc Tukey test. PT: Prothrombin time, INR: International normalized ratio, aPTT: Activated partial thromboplastin time.

DISCUSSION

In the present study, it was observed that two different acute stress protocols (restraint and cold restraint stress) had no clear effect on aPTT from coagulation indicators in rats, increasing PT and INR values, reducing fibrinogen concentration, had no effect on D-dimer concentration. To the best of the authors' knowledge, this study was the first to compare the effect of two acute stress protocols (restraint and cold restraint) on coagulation indicators in rats. According to the "glucocorticoid cascade hypothesis of aging" which explains the relationship between aging and stress response, immobilization stress increases corticosterone production in both aged and young animals, aged rats cannot reestablish their levels for at least four hours, indicating deficiencies in the negative feedback control of the HPA axis (Sapolsky et al. 1983, Sapolsky et al. 1986).

The use of young rats in our study is important in terms of the healthy response of the negative feedback mechanism to acute stress.

The temporal measurements of the extrinsic and intrinsic routes of coagulation are, respectively, PT and aPTT. Of the studies that examined at coagulation times in individuals, two of them found that there was no change in aPTT under acute psychological stress (de Boer et al. 2007, Zgraggen et al. 2005), while the other reported a shortening of aPTT (von Känel et al. 2009). One research (von Känel et al. 2004) found no change in PT, another (de Boer et al. 2007) found a tendency toward shortening, and yet another (von Känel et al. 2009) found a considerable shortening. It has been documented that individuals exposed to acute mental stress tend to increase the level of D-dimer with an increase in the

level of some coagulation factors (FVII: C, FVIII: C, FXII: C, von Willebrand factor antigen (vWF)) and fibrinogen (Zgraggen et al. 2005). In another study on humans, it was reported that aPTT decreased and fibrinogen, FVIII: C, D-dimer, and PT values increased significantly in psychologically acute stress exposure (Austin et al. 2012). The most recent study (von Känel et al. 2009) included prothrombotic alterations throughout two stress sessions, whereas earlier studies only looked at reactions at one stress session, which may help to explain why these contradictory results were obtained. More strong physiological effects are more likely to result from aggregating data from numerous assessments than from just one (Kamarck et al. 2000). It is unclear how acute psychological stress induces enhanced coagulation (von Känel et al. 2009, von Känel et al. 2001). Stress-induced catecholamine spillover and altered adrenergic receptor activity are two possible mechanisms.

Although there are considerable variations in blood coagulation when compared to the human standard, rat strains are commonly used in coagulation research (Lewis et al. 1985). The most notable distinction between human and rat blood coagulation is that rats have short clotting times that are pathological in human blood (García-Manzano et al. 2001). It has been reported that activated partial thromboplastin time (aPTT), thrombin time (TT) lengthened, prothrombin time (PT) did not change, and fibrinogen concentration decreased in rats exposed to repeated cold stress (Hatu et al. 1991). Another study reported inhibition of platelet aggregation, extrinsic coagulation, and a reduction in fibrinogen and antithrombin III levels in young rats subjected to experimental acute vital stress (prey animal; snake). In addition, increased platelet count with decreased aggregation capacity, hypocoagulation via the intrinsic pathway of plasma hemostasis activation, signs of thrombinemia, and increased antithrombin III have been reported in aged rats (Bondarchuk et al. 2020). While some studies have shown that platelet function increases response to acute stress (von Känel and Dimsdale 2000), it has been reported that it has no significant effect on platelet aggregation in mice (Matsuhisa et al. 2014). It has been shown that acute stress (water immersion restraint and cold restraint) in rats may cause a decrease in platelet aggregation (Malyszko et al. 1994, Takeda et al. 1992). In dogs under anesthesia, acute hypothermia led to transient platelet hypoaggregability (Yoshihara et al. 1985). In our study, although acute stress did not affect aPTT and D-dimer levels, it increased PT and INR values. In addition, fibrinogen concentration decreased under restraint and cold restraint stress. The possible reason for this is that platelet function may be impaired due to acute stress application, as previously stated in the rat study (Takeda et al. 1992). Platelet hypofunctions have been reported in the specific change in the rhythm of stressed (Hata et al. 1992, Kawabata and Hata 1993) or immobilized (Loban'-Chereda and Novosel'tseva 1990) rats, and this is supported by our research. Another reason may be heparin which is secreted from the mast cells of rats under restraint stress (Umarova et al. 1997). Heparin increases blood anticoagulant potential in response to stress.

CONCLUSION

In conclusion, acute stress (restraint and cold restraint stress) in rats prolongs PT, increases INR, decreases fibrinogen concentration, and does not affect aPTT and D-dimer levels. Further studies on this topic are needed.

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Conflict of Interest: All authors have read and approved the study. There is no conflict of interest between the authors.

Authorship Contributions: The authors contributed equally to this study.

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