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Sıçanlarda Periferal Adropin Uygulamasının Hipotalamik Hipofizer Adrenal Aks Üzerine Etkisi

The Effect of Peripheral Adropin Application on Hypothalamic Pituitary Adrenal Axis in Rats

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Öz: Amaç: Hipotalamik hipofizer adrenal (HPA) aks, strese ve inflamatuvar faktörlere yanıt oluşturmak gibi birçok görevlere sahiptir. HPA aksı, primer stres yanıt sistemidir. Adropin, enerji homeostazıyla ilişkili gen tarafından kodlanan peptit yapılı bir hormondur. Bu araştırmada adropin hormonunun HPA aksı üzerindeki histopatolojik ve immünohistokimyasal etkileri incelenmiştir.
Gereç ve Yöntem: Çalışmada otuz iki (32) Wistar Albino erkek sıçan kullanıldı. Sıçanlar 4 eşit gruba ayrıldı (n=8). Kontrol grubuna herhangi bir uygulama yapılmadı ve sham grubuna adropin çözücüsü verildi. Adropin tedavi gruplarına 4 µg/kg ve 40 µg/kg dozlarında intraperitoneal olarak uygulandı. Çalışma 10 gün sürdü. 11. günde hayvanlar sakrifiye edildi ve ilgili doku örnekleri toplandı.
Bulgular: Adropin uygulanan gruplarda kortizol, adrenalin, noradrenalin ve serotonin düzeyleri azalırken, dopamin seviyeleri arttı. Melatonin seviyelerinde önemli bir değişiklik oluşmadı. İmmünohistokimyasal boyamanın bir sonucu olarak, CRH, adropin gruplarında diğer gruplara göre artış göstermiştir.

Anahtar Kelimeler — Adropin, HPA Aksı, Hormon, İnflamatuvar, Stres.

Abstract: Purpose: Hypothalamic pituitary adrenal (HPA) axis has many missions such as responses to stress and inflammatory factors. HPA axis is the primer stress response system. Adropin is a peptid structured hormone coded by energy homeostasis related gene. In this research biochemical, histopathologic and immunohistochemical effects of adropin hormone on HPA axis were examined.
Material and Method: Thirty two (32) Wistar Albino male rats were used. The rats were separated into 4 equal groups (n=8). The control group did not receive any applications; and the sham group was given adropin-dissolvent. Adropin was administered as intraperitoneal to the treatment groups at the doses of 4 µg/kg and 40 µg/kg. The study lasted 10 days. On the 11th day, the animals were sacrified, and relevant tissue samples were collected.
Findings: While cortisole, adrenaline, noradrenaline and serotonin levels decreased, contrary dopamin levels increased in adropin administered groups. There were no important changes on melatonin levels. As a consequence of immunohistochemical staining, CRH has shown increase in adropin groups compared to other groups.

Keywords — Adropin, HPA Axis, Hormone, Inflammatory, Stress.

INTRODUCTION

Hypothalamic pituitary adrenal (HPA) axis has many missions such as responses to stress and inflammatory factors (1). It has three parts; hypothalamus, pituitary and adrenal gland. The HPA axis consists of three types of cells and hormones which are released from them: 1) Corticotropin releasing hormone (CRH) released from paraventricular nucleus (PVN), 2) Adrenocorticotropic hormone (ACTH) released from endocrine cells (corticotrophs) in the anterior pituitary and 3) Cortisol and/or corticosterone (CORT) which are glucocorticoid hormones released from endocrine cells in the zona fasciculata region of the adrenal cortex (2).

CRH is the main component of the HPA axis. ACTH stimulates the production and secretion of the steroid hormones such as glucocorticoids (3). Glucocorticoids are hormones, which are synthesized and released by the adrenal gland (4).

One feature of the activation of the HPA axis is that it provides adrenaline secretion from the adrenal medulla (5). Adrenaline stimulates central afferents, which are associated with locus coeruleus to induce the release of noradrenaline in various brain areas (6). Noradrenaline provides HPA activation by stimulating cells containing CRH in hypothalamic PVN (7).

The D1 and D2 receptors in the medial prefrontal cortex in the dopamine system modulate the HPA axis activation (8, 9). Serotonin (5-hydroxytryptomine or 5-HT) has a stimulatory effect on the HPA axis (10). Melatonin has shown an inhibitory effect on ACTH in human adrenal gland (11).

Adropin (Adr) was discovered by studies conducted in mice by Kumar et al. (12, 13). It is encoded by Enho, which is expressed in the liver and brain (12). In addition to pancreas, liver, brain and kidney tissues, it also has been shown in endocardium, myocardium and epicardium (14). The presence of Adr in the central nervous system suggests that it has a function as a neuropeptide. Furthermore, the role of autocrine/paracrine is also possible (15).

We investigated the effects of peripheral Adr on the HPA axis by applying it on rats. In the literature, we have determined that Adr is examined in studies such as energy metabolism, blood pressure, preeclampsia, polycystic ovary, breast cancer etc, yet no study about the effects on hypothalamic pituitary adrenal axis has been found.

Therefore, instead of making a stress model on HPA axis, as a starting point, we have searched what kind of effects will be seen by administering Adr hormone under normal circumstances and by examining some important hormones and tissues, which act in this axis, biochemically and histopathologically. In this respect, we believe that the results to be obtained from this research will provide both new and useful information for the literature in terms of representing a first and investigating the effects of Adr hormone on different areas.

2. MATERIALS AND METHODS

Atatürk University Experimental Animal Research and Application Center (ATADEM), Atatürk University Faculty of Medicine and Atatürk University Veterinary Faculty laboratories have been used in order to carry out the study.

The research was approved by Atatürk University Ethics Committee of Experimental Animals (19.04.2016 dated and decree no.2). All procedures in the experiment were carried out in accordance with the protocol in the ethics committee.

2.1 Animals

32 Wistar Albino male rats (Experimental Animal Research and Application Center of Ataturk University, Erzurum, Turkey) were used in the experiment. Their average weight was 300±15 g. Animals were randomized into four groups (n = 8); control group, sham group (300 µl-i.p.) pure water, low dose Adr (4 µg/kg-i.p.) group and high dose Adr (40 µg/kg-i.p.) group.

During the experiment, the animals were kept at 21±1 ° C medium. The experimental medium was prepared as 12 hours light and 12 hours dark. Animals were fed as *ad libitum* and standard rat feed was used. Tap water was preferred as drinking water.

2.2 Preparation and Application of Adr

Adr hormone obtained from Phoenix pharmaceuticals (USA) was used in this experiment. It was maintained at -20 ° C under appropriate conditions until the administration was carried out. At the beginning of the experiment, the amount to be used in each injection was dissolved in pure water.

The injection dose was 300 µl for a 300 g animal. Sham group was treated with 300 µl of Adr solvent (pure water). In low and high dose Adr groups, 4 µg/kg and 40 µg/kg, respectively, Adr hormone was administered by dissolving in pure water. Injections were administered as intraperitoneal (i.p.) for all groups at the same period intervals each day.

2.3 Completing the Experiment and Taking Samples

After 10 days of application, the animals were sacrificed. Adrenal gland, hippocampus, hypothalamus and blood samples were taken. Blood samples were centrifuged at 5000 rpm for 10 min and maintained at -80°C until the Enzyme-Linked ImmunoSorbent Assay (ELISA) kit analysis was carried out. Tissue samples taken from the hippocampus, hypothalamus and adrenal gland were kept in 10% formaldehyde solution for histopathological and immunohistochemical studies.

2.4 Hormone Analysis

Melatonin, serotonin, dopamine, cortisol, adrenaline and noradrenaline kits (Elabscience, Chinese) were used for hormone analysis. The kits were analyzed in ELISA reading device (ELISA, BioTEK powerwawe XS Winooski, U.K) by following the company protocols.

2.5 Histopathological Procedures

Adrenal tissues were detected in a 10% buffered formaldehyde solution. The tissues were washed in tap water and the formaldehyde was removed. Subsequently, routine tissue follow-up was performed (Shandon citadel 2000, Thermo). The followed tissues were embedded in paraffin and 5 μm sections were taken from these paraffin blocks with microtome (Leica RM2255/England) put to normal and polylysine slides.

Tissues taken to the normal slides were stained with hematoxylin eosin and (Olympus BX51, camera attachment DP72) was evaluated with light microscope as no (-), mild (+), moderate (++) and severe (+++) depending on the presence of the lesion.

2.6 Immunohistochemical staining

After deparaffinization of the tissues taken on the polylysine slide had been completed, it was kept for 10 min in 3% H_2O_2 to inactivate the endogenous peroxidase and was washed in Phosphate Buffer Saline (PBS). Then it was kept for 10 min at 500w in antigen retrieval solution to reveal the antigens in the tissues and washed in PBS. To prevent nonspecific binding, protein block solution was added and washed in PBS. Corticotropin releasing factor (CRF) Antibody (H-104) (Santa Cruz, Cat. No: sc-10718) was applied at 1/100 dilution ratio as primary antibody to PBS washed sections. Afterwards, the procedure specified by the Expose mouse and rabbit specific HRP / DAB detection IHC kit (abcam: ab80436) was followed. 3,3’ diaminobenzidine chromogen was used and was stained contrastly with Mayer’s hematoxylin. Positive cells were examined at 20x magnification in light microscope.

2.7 Statistical analysis

Statistical analysis of biochemical data was performed using IBM SPSS statistics 20.0 program. The normality of the obtained data was evaluated statistically. Hormone levels taken from blood plasma were evaluated by Bonferroni correction one-way variance analysis. The values were presented as mean ± standard error. $p<0.05$ was considered as statistically significant.

SPSS 16.0 program was used for statistical evaluation of histopathological data. Kruskal Wallis test was used to determine the between-groups difference of the data obtained semiquantitatively in histopathological examination, Mann Whitney U test was used to determine the groups, which form the difference. $p <0.05$ was considered as statistically significant.

3. RESULTS

3.1 Biochemical Results

The cortisol levels varying with i.p. Adr administration are shown in Figure 1. Plasma cortisol level has decreased in the high dose Adr group compared to the control group (* $p<0.05$, n=8).

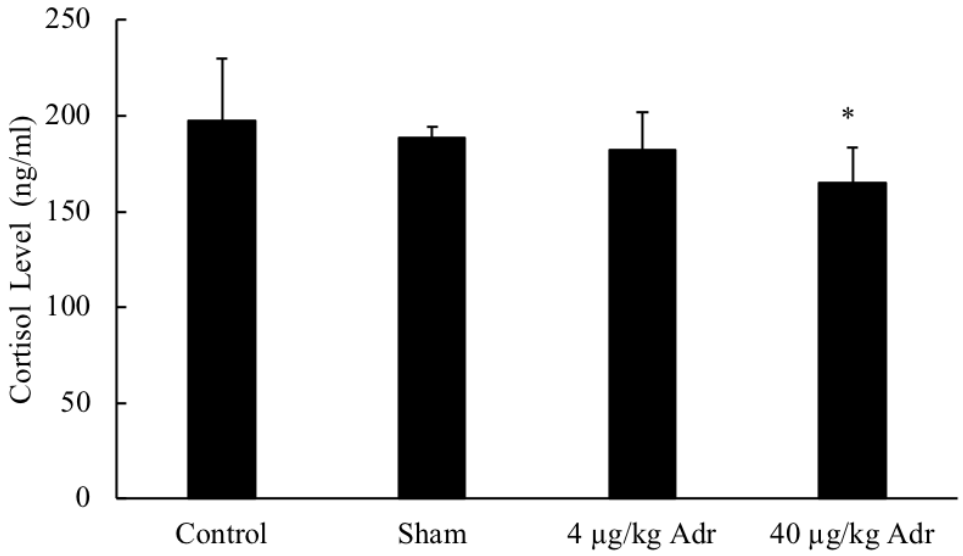

Figure 1 Effects of Adr administration on cortisol levels. Bonferroni correction one-way analysis of variance has been used to evaluate the data. Values have been presented as mean ± standard error (* $p <0.05$, n = 8).

In Figure 2, adrenaline levels, which changed with Adr administration, are shown. Plasma adrenaline levels have decreased in the high dose Adr group compared to the control group (* $p<0.05$, n=8).

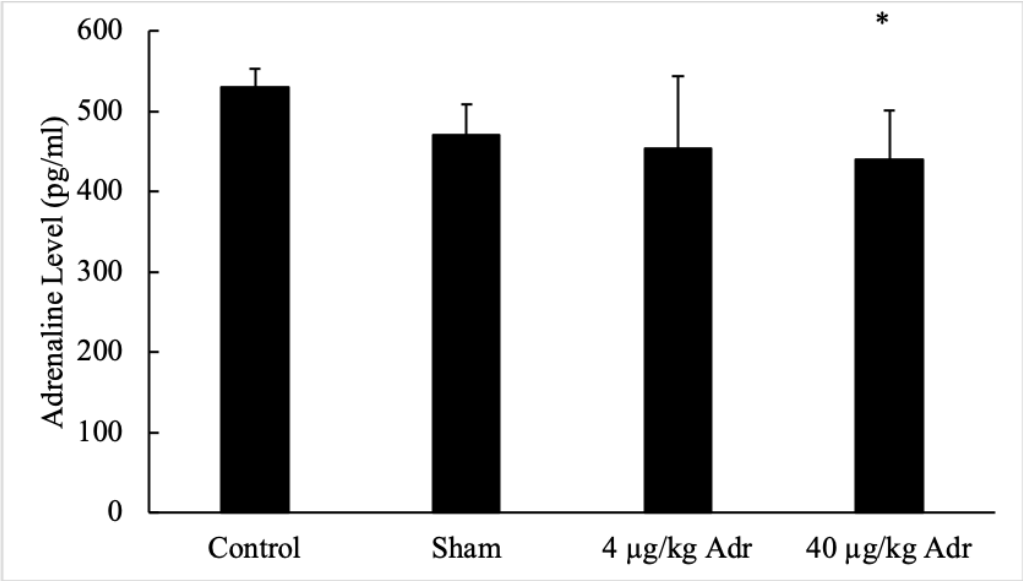

Figure 2 Effects of Adr administration on adrenaline levels. Bonferroni correction one-way analysis of variance has been used to evaluate the data. Values have been presented as mean ± standard error (* $p < 0.05$, $n = 8$).

In Figure 3, varying noradrenaline levels with Adr administration are shown. Plasma noradrenaline levels have decreased in the high and low dose Adr groups compared to the control and sham groups (* $p<0.01$, $n=8$).

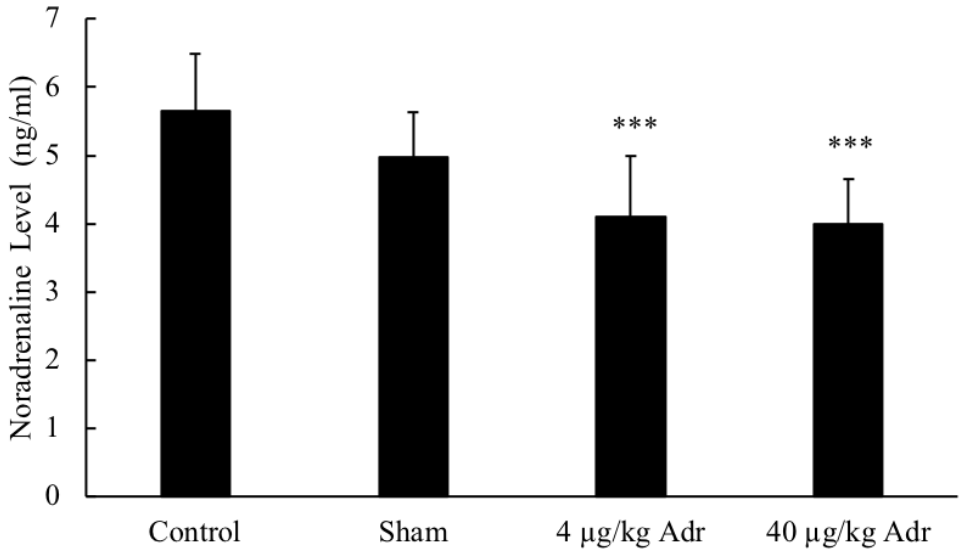

Figure 3 Effects of Adr administration on noradrenaline levels. Bonferroni correction one-way analysis of variance has been used to evaluate the data. Values have been presented as mean ± standard error (* $p < 0.001$, $n = 8$).

The dopamine levels varying with Adr administration are shown in Figure 4. Plasma dopamine levels have increased in high-dose Adr group compared to sham and low-dose groups (* $p<0.05$, n=8).

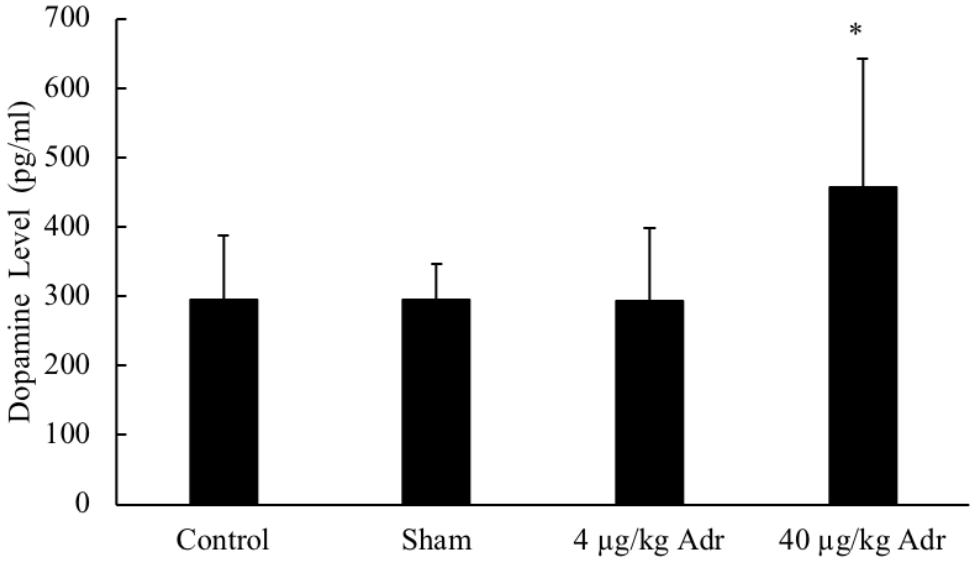

Figure 4 Effects of Adr administration on dopamine levels. Bonferroni correction one-way analysis of variance has been used to evaluate the data. Values have been presented as mean ± standard error (* $p<0.05$, n = 8).

Figure 5 includes serotonin levels changing with Adr administration. Plasma serotonin levels have decreased in high and low dose Adr groups compared to control and sham groups (* $p<0.05$, n=8).

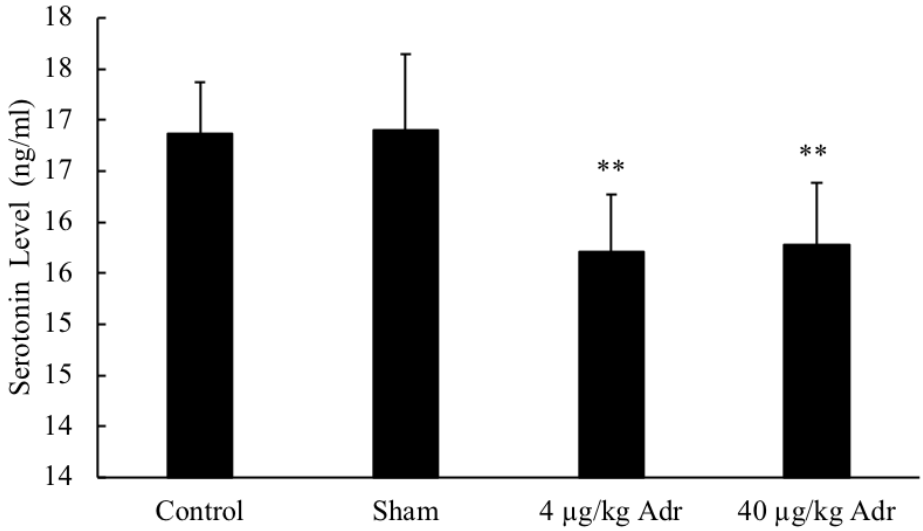

Figure 5 Effects of Adr administration on serotonin levels. Bonferroni correction one-way analysis of variance has been used to evaluate the data. Values have been presented as mean ± standard error (* $p<0.05$, n = 8).

The levels of melatonin, which are changed by Adr administration, are shown in Figure 6. No significant change has determined among the groups (n=8).

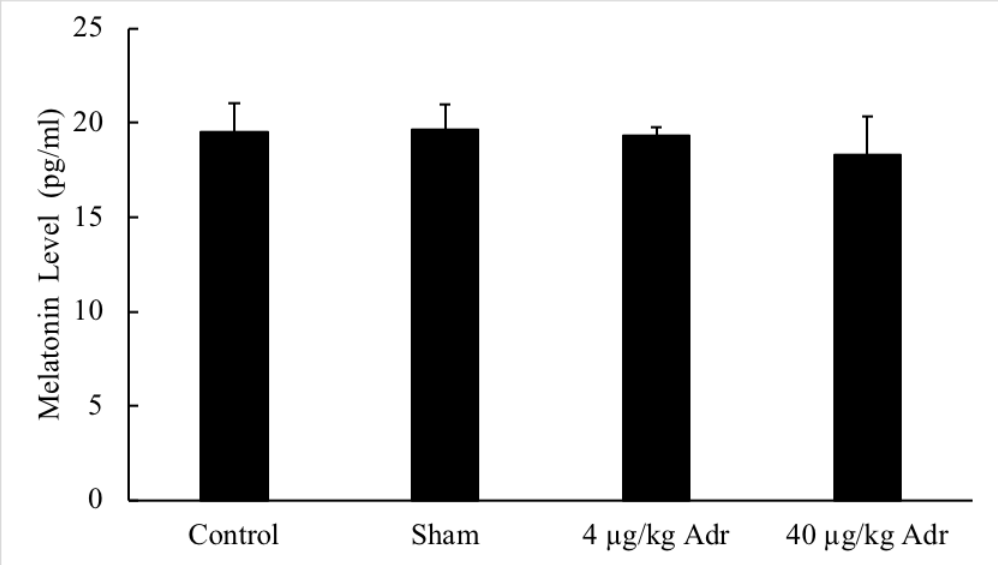

Figure 6 Effects of Adr application on melatonin levels. Bonferroni correction one-way analysis of variance has been used to evaluate the data. Values have been presented as mean ± standard error (n = 8).

3.2 Histopathological Results

Here, which the effect of Adr on adrenal gland tissue has been examined, the adrenal glands are observed to have normal histological structure in the control and sham groups (Figure 7.1., Figure 7.2). Severe hydropic degeneration has been observed in adrenal cortex cells in low-dose and high-dose Adr-treated groups (Figure 7.3., Figure 7.4.).

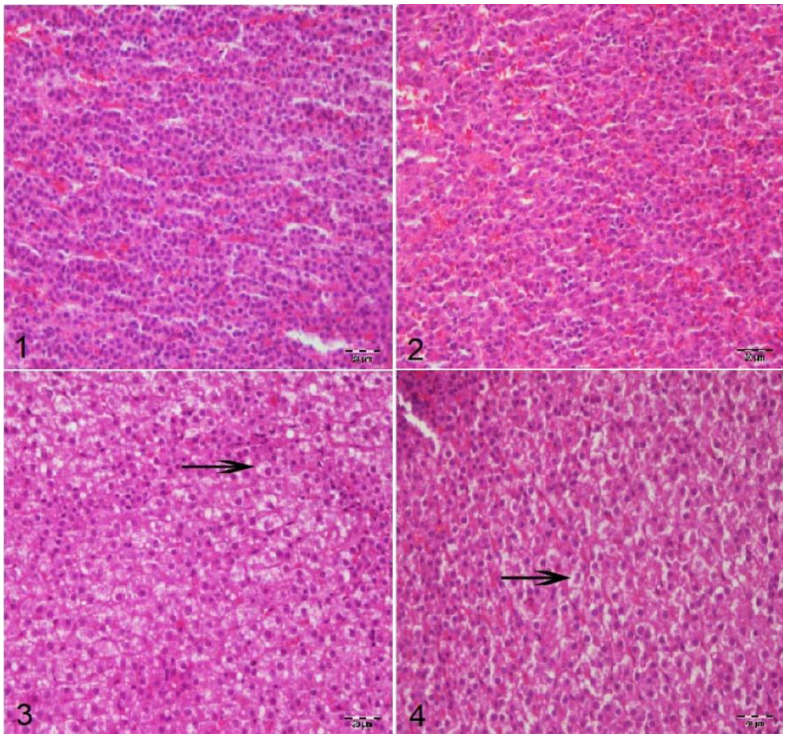

Figure 7 Effects of Adr application on adrenal gland tissues, 1. Control group. Normal histological appearance, 2. Sham group. Normal histological appearance, 3. 4 µg/kg Adr group. Severe hydropic degeneration in the adrenal cortex cells, (arrow) 4. 40 µg/kg Adr group. Severe degeneration of adrenal cortex cells (arrow)

Figure 8 shows the effects of Adr administration on the hippocampus and hypothalamus. As a result of immunohistochemical staining, an equal level of CRH immunopositivity has been found in the hippocampus region in the glial cells of all groups (Fig 8a, c, e, g). CRH has been observed moderately high in the neurons of the hypothalamus in the control group (Fig 8b). In the sham group, a mild immunopositivity has been determined (Fig 8d). Increase in CRH immunopositivity is observed in low and high doses of Adr compared to other groups (Fig 8f, h). The most intense immunopositivity has been observed in high dose administration of Adr (Fig 8h).

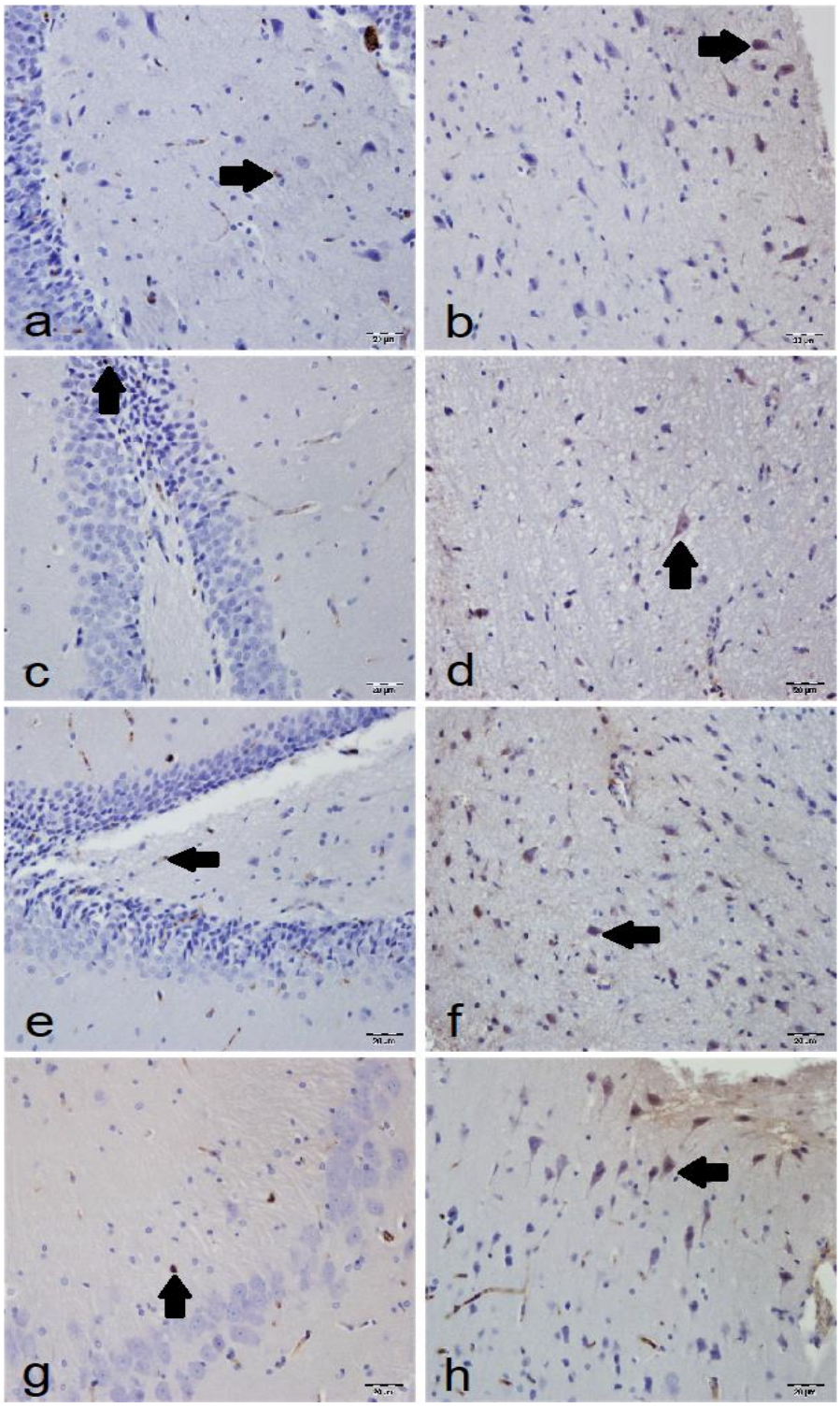

Figure 8 a) Control group, CRF immunopositivity in glial cells, arrow b) Control group, moderate CRF immunopositivity in neurons in the hypothalamus region, arrow c) Sham group, CRF immunopositivity in glia cells, arrow d) Sham group, mild CRF immunopositivity in neurons in hypothalamus region, arrow e) 4 µg/kg Adr, CRF immunopositivity in glia cells, arrow f) low dose of Adr, intense CRF immunopositivity in neurons in the hypothalamus region, arrow g) high dose of Adr, CRF immunopositivity in glia cells, arrow h) 40 µg/kg Adr, intense CRF immunopositivity in neurons in the hypothalamus region, arrow. (IHCx40)

3.3 Statistics Results

In histopathological examination, a significant difference was observed among the high and low dose group and the control and sham group in terms of degenerative changes (Table 1, $p<0.05$). No significant difference was observed between high dose and low dose groups (Table 1).

Table 1 Degenerative changes in the adrenal gland as a result of Adr application. Different letters (a, b) in the same column indicate intergroup differences ($p <0.05$).

	Hydropic Degeneration
Control	0.60 ± 0.24^{a}
Sham	0.80 ± 0.37^{a}
4 µg/kg Adr	2.20 ± 0.37^{b}
40 µg/kg Adr	2.40 ± 0.40^{b}

4. DISCUSSION

Adr, which is composed of Latin "adura'' (to throw into fire) and bir 'pinquis' (fats or oils), is a hormone which has a role in energy homeostasis (12). Adr is a newly discovered neuropeptide that is synthesized and released essentially from the brain and liver in rodents (16). Li et al. have observed that Adr regulates cardiovascular function and shows a protective effect against the development of cardiovascular diseases (17). In their study of dietary-induced obese mice, Gao et al. have found that Adr increases glucose tolerance, improves insulin resistance, and provides the primary use of carbohydrates (18). Shahjouei et al. reported that Adr is associated with many central nervous system diseases (15).

In our research, the levels of cortisol, adrenaline, noradrenaline, dopamine, serotonin and melatonin hormones were investigated in order to evaluate the effect of Adr on the HPA axis in rats. Tissue samples from hippocampus, hypothalamus and adrenal gland were examined.

Cortisol is one of the most widely used glucocorticoids as HPA axis activity biomarker in the studies (19). Cortisol, the last product of the HPA axis, is a steroid structured hormone, which is the major component of the stress response system (20). In a search of Herane-Vives et al., cortisol levels were found higher in individuals with major depressive episodes compared to healthy subjects in a 15-day period (21). In a research conducted by Barugh et al., cortisol levels were observed high in patients with stroke, for at least 7 days after stroke (22). In results, a decrease has been found in plasma cortisol level of the 40 µg / kg Adr group compared to the control group. Decrease of cortisol level as a result of Adr administration

may reduce the effectiveness of the HPA axis response by preventing the elevation of cortisol level in an HPA axis response against both physical and mental based stress.

Hypothalamic activation leads to adrenaline and noradrenaline release from the adrenal medulla (23). Adrenalin and noradrenaline released from the adrenal medulla constitute the fastest response to stress (24). Dhabhar et al. have reported a reduction not only in the production of catecholamines, but also in cortisol, in the rat study in which they performed adrenalectomy (25). We have found that plasma adrenaline level decreases in the 40 mg/kg Adr administrated study group compared to the control group, and plasma noradrenaline levels decrease in groups with 40 μg/kg Adr and 4 μg/kg Adr compared to the control and sham groups. As a result of Adr administration, a decrease in adrenaline and noradrenaline levels may lead to a reduction in the efficacy of the sympathetic response to exposure to any stress. In addition, the decrease in these hormones, which are also involved in stimulating the HPA axis, may cause a reduction in the HPA axis response level which will constitute a response to the stressor.

When a healthy person is exposed to physical stress, a saliva cortisol response forms positively associated with the level of dopamine release in the ventral striatum (26). Butts et al. have observed that glucocorticoids play a role in dopaminergic modulation in the brain (27). We found that, plasma dopamine levels were increased in 40 μg/kg Adr group compared to control, sham and low dose groups. An increase in dopamine levels as a result of Adr administration may have a positive effect on the HPA axis response.

Systemic administration of 5-HT 1A receptor agonists in rodents and humans has increased plasma ACTH and glucocorticoid production (28-31). Zhang et al. have found that while serotonin regulates the HPA axis, corticosteroids also regulate the serotonin synthesis (32). In results, plasma 5-HT levels have decreased in 40 μg/kg Adr and 4 μg/kg Adr groups compared to the control and sham groups. Decrease in 5-HT levels, as a result of Adr administration, may lead to a reduction in stimulation of the production of hormones such as ACTH and cortisol, which act in the HPA axis, and cause a reduction in the HPA axis response.

Melatonin has inhibited the glucocorticoid response to ACTH in non-human primates, sheep and rats by affecting the adrenal gland directly (33-35). Campino et al. have observed that melatonin has an inhibitory effect on ACTH in human adrenal gland (11). During our research, no significant change has been found in melatonin levels between 40 μg/kg Adr and

4 μg/kg Adr administrated groups. Since no significant change has been found in melatonin levels we think that Adr through this hormone has no effect on the HPA axis.

CRH, one of the biomarkers in stress studies (36), is one of the most necessary components in vertebrates in response to stress (37). When Yadawa et al. applied i.p. herbicide paraquat to the rats; they found that HPA axis response increased against stress due to the increase in CRH amount (38). In results, all groups CRH immunopositivity was found equal in glia cells immunohistochemically. However, CRH immunopositivity of the neurons in the hypothalamus region was found higher in 40 μg/kg and 4 μg/kg Adr administered groups compared to the control and sham groups. CRH immunopositivity in the 40 μg/kg Adr group was more intense than the 4 μg/kg Adr group. Although no significant change was found in the intensity of CRH in the hippocampus neurons, it was thought that the increase in CRH intensity in Adr-administrated groups in the hypothalamus would have a positive effect on CRH, the basic modulator of HPA axis and would affect the HPA axis response positively through this hormone.

In the tissue histopathological examination of the adrenal gland in 40 μg/kg Adr and 4 μg/kg Adr administrated group, degeneration was found in the cells compared to the control and sham groups. It was thought that this degeneration could lead to disruption in the adrenal gland step of the HPA axis response in Adr administration.

With this research, which we investigated the effects of Adr hormone on HPA axis, we have revealed that Adr, which causes decrease or increase in some of the hormones acting in the HPA axis response, may have an effect on HPA axis and we have also stated the effects on various tissues. As a result of Adr administration, while an increase has been found in CRH levels, which is the first step of HPA axis response, a decrease has been determined in cortisol, adrenaline and noradrenaline released from the adrenal gland. At the same time, there has been an increase in dopamine and a decrease in serotonin. However, no significant change has been observed in melatonin.

Although we think that Adr increases the amount of CRH, since we have not found any information in the literature whether the blood has passed the brain barrier, and that we have administered Adr as i.p, it is not clear if the increase in the amount of CRH is due to the effect of Adr on the brain. We also think that the decrease in the amount of cortisol as a result of adrenal gland degeneration contributes to an increase in the amount of CRH by having a negative feedback effect.

However, when the raise in the amount of dopamine is considered, increase in the amount of dopamine while expecting a decrease parallel to the reduction in the amount of cortisol suggests that Adr may lead an increase in dopaminergic activity by passing the blood brain barrier. In addition, this decrease in the amount of cortisol suggests that it may be due to a decrease in serotonin levels. It has been surmised that the decrease in the amount of serotonin can be directly related to the decrease in cortisol level due to Adr or the degenerative changes caused by Adr in the adrenal gland.

Even though the data we have obtained are limited to biochemical, histopathological and immunohistochemical examinations, it is important for us as it represents a first in our further studies in terms of giving idea and sheding light. Although Adr has been examined in many studies in the clinic, this research is very precious since it deals with the investigation of the effects of Adr on the HPA axis and especially the degenerative changes in the adrenal gland for the first time, and it draws attention about the possible changes in the adrenal gland in the long-term use of Adr.

5. CONCLUSION

Adr has showed a variable performance due to effect on HPA axis by increasing dopamine value while decreasing cortisol, adrenaline, noradrenaline, serotonin levels and not affecting melatonin value.

6. Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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