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# Possible Activation of the Immune System by Chronic Peripheral Nesfatin-1 Application at the Acute Phase of Ischemia/Reperfusion Injury

ORIGINAL INVESTIGATION

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ABSTRACT

**Objective:** Organ transplantation is one of the clinical scenarios involving ischemia and reperfusion process. Ischemia/reperfusion is the pivotal mechanism of organ injury during transplantation. Thus, ischemia/reperfusion (I/R) injury is a biphasic phenomenon that can damage the graft by inflammatory responses. The hypothalamic-pituitary-adrenal (HPA) axis is the main hormonal system that is activated under the influence of stress. Normal HPA axis activity leading to the release of glucocorticoids is essential for homeostasis and survival during stress. Cortisol, a key controller of stress response, is released by the HPA axis. The disrupted release of cortisol in response to inflammation has been shown in animal models. Nesfatin-1 is a peptide involved in the regulation of homeostasis and has anti-inflammatory as well as anti-ischemic properties. Therefore, we aimed to identify the effect of chronic peripheral nesfatin-1 application on the plasma level of cortisol in a rat model of intestinal I/R-based stress.

**Materials and Methods:** Two-month-old 28 Wistar Albino male rats that weighed an average of 200–250 g were used and were randomly divided into the following four experimental groups (n=7): laparotomy, I/R, nesfatin-1+laparotomy, nesfatin-1+I/R. Blood samples were collected in tubes with EDTA. Plasma cortisol levels were analyzed by rat enzyme-linked immunosorbent assay (ELISA) kits.

**Results:** Statistically significant decrease was found in the plasma level of cortisol in nesfatin-1+I/R group compared with I/R group (p=0.026)

**Conclusion:** Nesfatin-1 application can inhibit anti-inflammatory responses under the early phase of intestinal I/R and support immune reactions by reducing plasma cortisol level. This effect of nesfatin-1 may also increase the rejection of grafts during transplantation period.

Keywords: Ischemia/reperfusion injury, nesfatin-1, inflammation, cortisol, hypothalamic-pituitary-adrenal axis, transplantation

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## INTRODUCTION

Stress is a physical, chemical, or an emotional factor to which an organism fails to make a satisfactory adaptation (1). The hypothalamic-pituitary-adrenal (HPA) axis is the main hormonal system that is activated under the influence of stress; acute stress causes a marked increase in glucocorticoid (GC) concentration in blood through the activation of this system (2). Multiple systems including immune and cardiovascular systems as well as several hormones such as adrenocorticotrophic hormone (ACTH), GCs, and catecholamines are involved in stress response (3). Eventually, normal HPA axis activity leading to the rhythmic and episodic release of adrenal GCs is essential for homeostasis and survival during stress (4).

Injury caused by oxidative stress occurs in many clinical scenarios involving ischemia and reperfusion such as organ transplantation, hemorrhagic shock, myocardial infarction, and cerebral vascular events (5). Because ischemia initiates the injury and reperfusion worsens the ischemic injury owing to inflammatory responses induced by endothelial factors. Subsequently reperfusion triggers cell apoptosis and necrosis, although it is essential for the survival of ischemic graft (6, 7). Finally, the primary tissue damage occurs during the reperfusion phase (8). At present, it is known that ischemia/reperfusion (I/R) injury is a situation triggering the inflammatory, apoptotic, and immune mechanisms, leading to a stress response in the organism.

Cortisol, the most important human GC, is released by the HPA axis in response to inflammation, stress, and other types of stimuli (9-11). In animal models, the disrupted release of cortisol in response to inflammation was shown, which is of great importance (12).

Recently, Oh-I et al. (13) described a new anorexigenic protein which is derived from nucleobindin 2 (NUCB2) and named it NUCB2-encoded satiety and the fat-influencing protein-nesfatin- (14). Nesfatin produces three major peptide products such as nesfatin-1 (spanning residues 1–82), nesfatin-2 (residues 85–163), and named it NUCB2-encoded satiety and the fat-influencing protein as nesfatin (15). Nesfatin-1 is involved in the regulation of homeostasis and has anti-inflammatory, anti-apoptotic, and anti-ischemic properties (13, 16, 17).

Based on the literature review, in our study, an intestinal I/R-based stress model was created to evaluate the effect of chronic peripheral nesfatin-1 application on plasma cortisol level in rats.

## MATERIALS and METHODS

### Animals and Experimental Conditions

All experimental protocols conducted on animals were consistent with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23) and were approved by the Dumlupinar University Ethics Committee of Animal Care and Usage. In this study, 28 two-month-old Wistar albino male rats were used that weighed an average of 200–250 g. They were reared under the supervision of a veterinarian and were kept in a well-ventilated, noiseless environment, and allowed free access to food and water. The rats were housed in a room with controlled temperature (23±1°C) and relative humidity (50%±5%), and they were kept in transparent plastic cages (42×26×15 cm), each containing three or four rats that were exposed to a 12:12 light–dark cycle.

### Experimental Design

The rats were randomly divided into four experimental groups (in each group, n=7). The groups were as follows: 1) sham rats underwent laparotomy (L), 2) I/R rats underwent the occlusion of the superior mesenteric artery for 30 min followed by 2-h reperfusion (18), 3) nesfatin-1+L rats were treated with intraperitoneal nesfatin-1 (i.p.; 0.25 nmol/g) (Phoenix Pharmaceuticals, Catalog No: 003-22B) for 10 consecutive days (19) and underwent laparotomy (N+L), 4) nesfatin-1+I/R rats were treated with i.p. nesfatin-1 (0.25 nmol/g) for 10 consecutive days and underwent the occlusion of superior mesenteric artery for 30 min followed by 2-h reperfusion (N+I/R).

### Blood Samples and Measurements

At the end of the experimental period, all animals were anesthetized with ketamin/xylazine HCl (75 mg/kg/10 mg/kg i.p.). Blood samples were collected in tubes with EDTA. After centrifugation, plasma samples of each rat were stored at −80°C until ELISA analysis. Plasma cortisol levels (Cusabio Biotech, Cat No CSB-E05112r) were analyzed by rat ELISA assay kits. First, we set up a blank well without any solution and added 50 µl of standard and sample as well as 50 µl antibody to each well, except the blank well. After that plate was mixed well for 60 s, it was incubated for 40 min at 37°C. After incubation, each well was washed three times with wash buffer (200 µl) using an autowasher. After the last wash, any remaining wash buffer was removed by aspirating. Horseradish peroxidase (HRP)-conjugate (100 µl) was immediately added to each well, except the blank well. The plate was incubated for 30 min at 37°C. Washing process was repeated for five times. A total of 90 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to each well and incubated for 20 min at 37°C. Subsequently, 50 µl of stop solution was added to each well. Concentration of each sample was calculated according to their chemiluminescence data. Chemiluminescence data were analyzed by an ELISA microplate reader (das, Digital and Analog Systems; Vimercate, MI, Italy).

### Statistical analysis

Statistical analyses were conducted by Statistical Package for Social Sciences (SPSS, Inc.; Chicago, IL, USA) 16.0 package program. All data were given as mean ± standard deviation (SD). Statistical significances among all groups and between the two groups were analyzed by Kruskal–Wallis and Mann–Whitney U tests, respectively. Differences were considered to be statistically significant at $p<0.05$.

## RESULTS

### Plasma Cortisol Levels

Statistically significant difference was found for the plasma cortisol level among the L (6.83±0.14 ng/mL), I/R (7.3±0.083 ng/mL), N+L (7.19±0.07 ng/mL), and N+I/R (7.15±0.05 ng/mL) groups ($p=0.009$). The plasma cortisol levels in I/R and N+L groups were significantly higher than those in the L group; $p=0.004$ and $p=0.038$, respectively (Figure 1). A statistically significant decrease was found in the plasma cortisol level in N+I/R group compared with the I/R group ($p=0.026$) (Figure 1).

## DISCUSSION

Ischemia/reperfusion is a pathological condition characterized by an initial restriction of blood supply to an organ, followed by the restoration of perfusion and concomitant reoxygenation. Occlusion of the arterial blood supply results in severely impaired metabolic supply and demand, leading to tissue hypoxia (20, 21). Furthermore, despite the successful reopening of the vascular supply system, an ischemic organ may not immediately regain its perfusion (22). Subsequent restoration of blood flow and reoxygenation is associated with an exacerbation of tissue injury and a strong inflammatory response, which is called as "reperfusion injury" (20, 21). At present, it is known that ischemia and reperfusion leads to the activation of cell death programs, including apoptosis and necrosis, and that reperfusion injury is characterized by autoimmune responses associated with the activation of innate and adaptive immune responses and inflammatory cell trafficking into the diseased organ (22-24). Moreover, I/R is the pivotal mechanism of the organ injury during transplantation and is a multifactorial

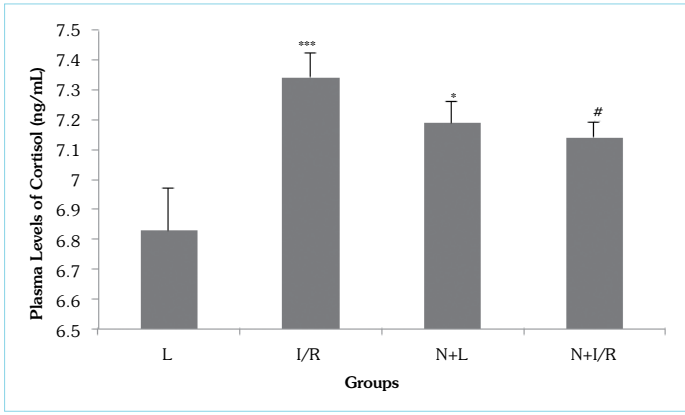


**Figure 1. Plasma cortisol levels in L, I/R, N+L, and N+I/R groups**
L: Laparatomy; I/R: Ischemia/reperfusion; N+L: Nesfatin-1+Laparatomy; N+I/R: Nesfatin-1+ischemia/reperfusion
*: The significance between L and the other group; $p<0.05$ (Mann–Whitney U test)
#: The significance between I/R and the other group; $p<0.05$ (Mann–Whitney U test)
***: The significance between L and the other group; $p<0.001$ (Mann–Whitney U test)

process that affects graft function after transplantation (25, 26). The reason is that transplantation is always associated with I/R injury as well as inflammation and rejection (27). By occurring early in the transplant process, I/R initiates a cascade of molecular and cellular events; consequently, acute and chronic changes develop, thereby influencing the structure and function of the organ that may contribute to reduced graft survival (28). Additionally, activation of the immune system as a result of the disturbances involved in I/R injury contributes to tissue and organ damage (5). Based on the literature, I/R injury initiates a chain of events including apoptotic, inflammatory, and immune mechanisms that trigger the stress response in the organism.

The HPA axis (neuroendocrine route, resultant release of GCs, namely cortisol) and the sympathetic nervous system (SNS, neural route, resultant release of catecholamines, namely noradrenaline (NA)/adrenaline and neuropeptides) are the two main pathways of the stress response (29). Dysregulation of either of these stress systems can lead to the dysregulation of several other physiological systems, including the immune system, which leads to a maladaptive stress response (29). Because the HPA axis and SNS interact with each other in a bidirectional manner and coordinate the responses of many other physiological systems to a stressor (29-31). Although these two systems act together, the HPA axis is the main center and the activation of this system provides the animal's ability for adaptation and coping with stress (32).

The end-point of the activation of the HPA axis is the release of GCs, i.e., cortisol in humans and corticosterone in rodents, from the adrenal cortex into the general circulation (33). The synthesis and release of GCs are controlled by ACTH, which is released from the anterior pituitary gland. The release of ACTH is regulated by corticotropin-releasing factor (CRF) and arginine vasopressin (AVP), both of which exert a synergistic action on the release of ACTH. In response to acute stress or inflammation, the HPA axis is activated, thereby evoking the release of CRF and AVP into the hypophysial portal blood; this results in an increased release of ACTH and cortisol into the blood (33). Additionally, the degree of the activation of HPA axis is closely related to the intensity of stress experienced by animals (2). It is known that rats are very sensitive to stress so that a mild stressor results in an excessive increase in GC levels (34). Because animals respond to a stressor by increasing their GC levels, several studies have been focused on measuring these hormone levels (35). Up to date, many different techniques including sampling from blood and feces have been used to measure GC concentrations. Consequently, blood GC concentrations have been used as an index of stress in several studies (36).

GCs are a class of steroid hormones that have a wide range of biological activities (37). GCs are secreted in response to inflammation and stress, and they potently suppress immunity and inflammatory responses (9, 10). Thus, GCs are widely recognized as the regulators of adaptive inflammation, immunity, and stress response in the organism (9, 38). Cortisol is the major stress hormone and the key controller of the stress response that regulates and supports various important immunologic, homeostatic, cardiovascular, and metabolic functions (11, 37).

In our study, rats were used to create an intestinal I/R-based experimental stress model; as an indicator of the stress and inflammatory response, plasma cortisol levels were measured. Statistically significant increase in the plasma level of cortisol was observed in the I/R group compared with L group. We suppose that this increase in cortisol level in the I/R group was a result of stress and inflammatory response, consistent with the findings in the literature. Although it may be advantageous to measure the plasma corticosterone level instead of cortisol level in rats, we measured the plasma cortisol level, which was a limitation of our study.

Nesfatin-1 is an 82-amino acid peptide that is involved in food restriction and in the regulation of homeostasis (13, 15, 39). Nesfatin-1 is localized in neurons of the hypothalamus and brain stem and is colocalized with stress-related substances such as CRF, oxytocin, proopiomelanocortin, NA, and 5-hydroxytryptamine (5-HT) (40). It is suggested that there is a link between nesfatin-1 and stress responses because the central nesfatin-1 system is stimulated by stress and activates CRH, NA, and 5-HT neurons as well as the HPA axis, thereby evoking both central and peripheral stress responses (40, 41). According to the literature, nesfatin-1 has homeostatic, anti-ischemic, anti-inflammatory, and anti-apoptotic properties (13, 16, 17). Thus, we aimed to investigate the effect of nesfatin-1 on the plasma level of cortisol in a rat model of intestinal I/R-based stress.

In a study by Merali et al. (42), it was reported that the intracerebroventricular (ICV) administration of nesfatin-1 potentiates stressor-induced increases in plasma ACTH and corticosterone levels in rats. Angelone et al. (16) suggested that nesfatin-1 protects the heart against I/R injury by reducing infarct size and releasing lactate dehydrogenase as well as by post-ischemic contracture. Özsavci et al. (17) reported that nesfatin-1 exerted a neuroprotective effect in subarachnoid hemorrhage-induced injury in rats by inhibiting neutrophil infiltration and the subsequent release of inflammatory mediators.

It has been shown that nesfatin-1 has anti-inflammatory effect under different conditions (17, 43). To our knowledge, we could not observe any reports about the effect of nesfatin-1 in laparotomy. In our study, as expected, a significant increase in the plasma cortisol level was observed in the N+L group compared with the L group by chronic i.p. nesfatin-1 application. In contrast, a significant decrease was observed in the plasma level of cortisol in the N+I/R group compared with the I/R group. Based on our findings, we can say that the release of cortisol was suppressed by nesfatin-1 application, particularly under stress conditions such as I/R. Interestingly, this finding indicated the dual effect of nesfatin-1, which means that nesfatin-1 behaves differently in different conditions. We suggest that there is a central inhibitor effect of nesfatin-1 on the release of cortisol, although we cannot explain the exact mechanism for the suppression of cortisol by nesfatin-1 in the N+I/R group. Also, we suppose that chronic peripheral nesfatin-1 application can inhibit anti-inflammatory responses under the early phase of intestinal I/R and support immune reactions by reducing plasma cortisol level. Because of the fact that I/R is an important part of organ transplantation, we hypothesize that this effect of nesfatin-1 also increases the rejection of grafts during transplantation period.

Finally, better understanding of the mechanisms involved in I/R injury provides a new perspective to the development of the

therapeutic strategies, which will improve the outcomes of transplantation. Here, we emphasized that nesfatin-1 may increase the rejection of grafts during transplantation period by inhibiting anti-inflammatory responses through the reduction of cortisol levels, although it is gaining attention as an anti-inflammatory agent. However, further experimental studies are essential to clarify the effect of nesfatin-1 on the immune system in different physiological and pathophysiological conditions.

## CONCLUSION

Chronic peripheral nesfatin-1 application can inhibit anti-inflammatory responses under the early phase of intestinal I/R and support immune reactions by reducing plasma cortisol levels. This effect of nesfatin-1 may also increase the rejection of grafts during the transplantation period.

**Ethics Committee Approval:** Ethics committee approval was received for this study.

**Authors' Contributions:** Wrote the paper: ÜT, CA. Conceived and designed the experiment: ÜT, CA, OG, RA. Performed the experiment and analyzed the data: ÜT, CA, OG, RA, SŞ. Critical revision of the article for important intellectual content: ÜT, CA, OG, RA, SŞ, GE, HAE, GT, ST. All authors have read and approved the final manuscript.

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