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EFFECT OF CHRONIC STRESS ON SERUM AND TISSUE LEVELS OF KLOTHO AND TNF-α

KRONİK STRESİN KLOTHO VE TNF-α'NIN SERUM VE DOKU SEVİYELERİ ÜZERİNE ETKİSİ

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ABSTRACT

Objective: Chronic stress is recognized as a factor that affects almost all organs and tissues and disrupts homeostasis. Stress also directly impacts the inflammatory process. In addition, klotho is a protein associated with lifespan. This study investigates the relationship that chronic stress has with klotho and tumor necrosis factor alpha (TNF-α), which is a key protein in the proinflammatory process.

Materials and Methods: The study involves 16 Wistar albino rats divided into control and stress groups. The study applies the protocol for the chronic unpredictable mild stress (CUMS) model and uses the enzyme-linked immunosorbent assay (ELISA) method to measure the TNF-α and Klotho levels in the serum, heart, aorta, and liver tissues.

Results: The serum samples obtained from the stress group were found to have a significant decrease in klotho levels (p=0.013) and high TNF-α levels (p=0.042). However, no significant difference was observed in the TNF-α or klotho levels in the heart, aorta, and liver tissues between groups.

Conclusion: Under chronic stress, a significant increase is observed in TNF-α, one of the main cytokines of inflammation, as well as a decrease in klotho hormone. This research endeavors to contribute to the existing body of knowledge concerning the interplay among stress, inflammation, and longevity.

Keywords: Chronic stress, inflammation, klotho, TNF-α

ÖZ

Amaç: Kronik stres hemen hemen tüm organları/dokuları etkileyen ve homeostazı bozan bir faktör olarak kabul edilmektedir. Stres, enflamatuar süreç üzerinde doğrudan bir etkiye sahiptir. Bununla birlikte, Klotho yaşam süresi ile ilişkili bir proteindir. Çalışmamızda kronik stres ile proinflamatuar süreçte anahtar bir protein olan Tümör nekroz faktör alfa (TNF-α) ve Klotho arasındaki ilişkiyi araştırdık.

Gereç ve Yöntemler: Çalışmamız kontrol ve stres gruplarına ayrılan 16 Wistar albino'dan oluşmuştur. Kronik öngörülemeyen hafif stres model protokolü uygulandı. Serum, kalp, aort ve karaciğer dokusundaki TNF-α ve Klotho düzeyleri ELISA yöntemi kullanılarak ölçüldü.

Bulgular: Stres grubundan alınan serum örneklerinin Klotho düzeylerinde anlamlı azalma (p=0,013), TNF-α düzeylerinde ise anlamlı yükseliş (p=0,042) olduğu belirlendi. Ancak; gruplar arasında kalp, aort ve karaciğer dokularındaki TNF-α ve Klotho düzeylerinde anlamlı bir fark gözlenmedi.

Sonuç: Kronik stres altında inflamasyonun ana sitokinlerinden biri olan TNF-α' da belirgin bir artış, Klotho hormonunda ise azalma gözlendi. Araştırmamız; stres, iltihaplanma ve uzun ömür arasındaki etkileşime ilişkin mevcut bilgi birikimine katkıda bulunmaya çalışmaktadır.

Anahtar Kelimeler: Kronik stres, inflamasyon, Klotho, TNF-α

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INTRODUCTION

Homeostasis denotes the intricate process of governing and upholding cellular equilibrium in response to external stimuli. Stress is recognized as a disruptive factor that affects homeostasis (1). Stressors can cause changes in intercellular communication pathways within the body (2). In cases of chronic stress, alterations in hormones, proinflammatory cytokines, and neurotransmitters can lead to morphological changes in organs, tissues, and arteries (3). These biological changes can result in the development of cardiovascular pathologies, metabolic disorders, neurodegenerative changes, and associated functional impairments (4, 5). The chronic stress process triggers adaptations for maintaining organismal homeostasis (2). These adaptive mechanisms govern the functioning of the hypothalamic-pituitary-adrenal (HPA) axis, which orchestrates the body's stress response. The release of mediator molecules through this pathway results in the restructuring of numerous biological structures at the cellular level, facilitating physiological adaptations to stress (6, 7).

Chronic stress has a direct impact on the inflammatory process (8). When looking at the pathogenesis of psychological stress and inflammation, they are noted to be parts of a mutually reinforcing cycle (3). During chronic psychological stress, the continuous active sympathetic discharge of the autonomic nervous system, metabolic response, gain in oxidative stress molecules, and activation of NF-kB constitute the basic pillars of the biological cascade (9). The correlation between psychological stress and leukocytes is of interest due to the effects stress has on the immune system. Psychological stress can cause the release of stress hormones such as cortisol and catecholamines (6). These hormones can affect the functions of leukocytes and the biomodulators that get released. Depression and stress can both directly and indirectly influence the secretion of proinflammatory cytokines (9).

Klotho is a protein associated with lifespan (10) and represents a transmembrane protein complex that assumes crucial functions in regulating the blood electrolyte balance (11). Klotho is a member of a biological axis along with FGF23 and is described as a cellular receptor coactivator (12). A substantial reduction is observed in the human population in serum concentrations of klotho after the age of 40, thus establishing it as one of the pivotal proteins associated with the aging process. Klotho is primarily synthesized in the body in the distal tubules, choroid plexus, and parathyroid gland (10-13). Kuro-o et al. observed overexpression of klotho gene expression levels using transgenic methods to result in a 30% longer lifespan in mice compared to the control group. Additionally, experiments that silenced klotho gene expression levels in mice led to rapid aging processes and systemic disorders such as cardiovascular disorders, neurodegeneration, and kidney failure (14, 15). The factors commonly associated with longevity, such as exercise, regular sleep, and balanced nutrition, are known to increase klotho levels (11). Meanwhile, chronic stress is widely accepted as a factor that negatively affects health and lifespan (16, 17). Moreover, the relationship between chronic stress and klotho, which is known to be a factor that causes cellular and systemic tissue damage, has recently become a research area that draws attention (13-17).

Cytokines are biomolecules that regulate inflammation. They play an active role in many processes such as the immune system, infections, and tissue damage (8). The correlation between klotho and cytokines is intricately linked to cellular senescence, inflammation, and various other biological processes. In this context, klotho provides protection against oxidative stress by increasing the expression levels of antioxidant enzymes (18). Proinflammatory cytokines elicit an elevation in cell adhesion proteins, whereas klotho is hypothesized to exert its anti-inflammatory effects through the downregulation of relevant adhesion proteins (19). When looking at the other side of the equation, experimental increases in oxidative stress lead to a significant decrease in klotho expression (20). Within this scope, an inverse relationship has also been stated to be observed between proinflammatory cytokines and klotho's association with longevity, thus indicating a negative correlation (21). Further investigation of this relationship may help develop new treatment options for various health issues such as aging and inflammation.

Chronic unpredictable mild stress (CUMS) is a type of stress caused by the stressors to which a person is regularly exposed but which are unpredictable and mild in nature (22). CUMS differs from the acute stress that occurs in response to a specific event or situation. Chronic unpredictable mild stress can occur due to the small but constant stresses a person experiences in their daily life, of which they are generally unaware, but from which constantly feel a sense of tension and anxiety (23). The objective of this study is to examine the impact chronic mild stress has on the expression of klotho and TNF-α. To this end, the study aims to determine the levels of target proteins in the heart, aorta, liver, and serum of rats subjected to chronic unpredictable stress and examine their relationship with each other. The study is of the opinion that the data acquired from this research can make a valuable contribution to unraveling the intricate biological mechanisms that underlie the impacts of chronic stress on the human body.

MATERIALS and METHODS

Animals and standard procedures

The study obtained Presidency approval (Approval No: 3/4-2023 Date: 29.03.2023) from Kocaeli University Animal Experiments Local Ethics Committee. The study uses 4-month-old female *Wistar albino* rats (n=16) weighing 190-200 gr. The rats were divided into two groups in standard cages 2 weeks before the start of the study process and provided standard room conditions. The rats' menstrual cycles were considered and analyzed. During anesthesia, a combination of ketamine hydrochloride (90 mg/kg; Ketalar, Parke-Davis) and xylazine hydrochloride (12 mg/kg, 2%; Rompun, Bayer) was administered intraperitoneally to the rats. Euthanasia was performed by cervical dislocation after intracardiac blood collection. The

Table 1. Stressors applied in the chronic unpredictable mild stress (CUMS) model

1. Cage inclination, 45°/24 hours	5. Changing day-night cycle
2. Hanging by the tail, 1 minute	6. Cage shaking, 10 minutes
3. Buoyancy in cold water 4°C/5 minutes	7. Cage wetting 200 mL/24 hours
4. Buoyancy in hot water 45°C/5 minutes	8. Exchanging sawdust between cages

study applied the principles from the Guidelines for the Care and Use of Laboratory Animals.

Stress model and groups

The study uses the CUMS protocol, which has been previously defined in the literature, as the stress model (22, 23). A total of eight different stressors were identified, and the order of the stressors was determined beforehand (Table 1). At the end of the experiment, anhedonic behaviors, which are considered an indicator of the depression status of animals, were closely monitored and measured.

During the 4th week of the experiment, a sucrose preference test was conducted to measure anhedonic behaviors in stressed animals. The test placed 2 water bottles in each cage and was applied for 6 days. One bottle contained 200 mL of a 20%-sucrose solution, and the other contained 200 mL of tap water. The rats were allowed to drink from both bottles during the first 5 days for habituation. The water bottles were changed every 12 hours. Sucrose consumption was calculated as the ratio of sucrose consumption to total consumption:

Sucrose Consumption=(Sucrose consumption x 100) / Total consumption

This study formed the rats into two groups (i.e., experimental and control groups) before applying the stressors to the experimental group for 28 days. The order in the application protocol was determined at random prior to the experiment.

Tissue lysis procedures

Analysis was performed on the heart, aorta, and liver tissues and serum. To eliminate any blood contamination, the tissues underwent washing with a saline solution containing 0.09% NaCl. Subsequently, the tissues were weighed and homogenized in a 1:10 ratio of phosphate-buffered saline (PBS) at a pH of 7.4. The homogenization was carried out at a speed of 24,000 revolutions per minute using the T25 Basic Ultra Turrax homogenizer (IKA Werke, Breisgau, Germany). Following homogenization, the samples were centrifuged at 10,000 times the force of gravity for 15 minutes at a temperature of 4°C. The resulting homogenate was then divided into smaller tubes and preserved for further analysis based on tissue-specific measurements.

ELISA method and biochemical procedures

Blood samples (3 ml) were taken from the left ventricle. Blood specimens were allowed to clot for two hours at room conditions and centrifuged for 15 minutes at a 1000g force at 4-8 ^{o}C. The supernatants were collected and diluted to a 1:10 ratio before the assay. Serum, heart, aorta, and liver tissues samples were stored at -40°C. Serum klotho and TNF-α levels were determined with the enzyme-linked immunosorbent assay (ELISA; Elabscience, Houston, USA) kits and measured with the Alisei Quality System Seac Radim Company Analyzer (Rome, Italy)-ELISA reader based on the manufacturer's instructions (catalog number E-EL-R2580 for klotho; Catalog number E-EL-R2856 for TNF-α). The results were multiplied by the dilution coefficient, and their concentrations were calculated according to the kit's standards. The ELISA kit has a sensitivity of 0.10 ng/mL and detection range of 0.16-10 ng/mL for klotho, while it has a sensitivity of 9.38 pg/mL and detection range of 15.63-1000 pg/mL for TNF-α. The modified Lowry method was employed to determine the protein content (24). The total protein concentration of the liver tissue was equalized prior to the ELISA process.

Data analysis and statistics

The Kolmogorov-Smirnov analysis was performed regarding the normal distribution suitability test in the statistical evaluation of the results. The independent T-test was applied for the normally distributed values. The Mann-Whitney U-test was used for the non-normally distributed values, with p-values of 0.05 or less being considered statistically significant. The package program SPSS 22.0 (IBM SPSS Corp., Armonk, NY, USA) was used for the statistical analyses, while the package program GraphPad Prism8 was used for designing the figures.

RESULTS

The serum klotho levels were 4.08±0.06 ng/ml for the control group and 3.72±0.11 ng/ml for the stress group. The serum TNF-α levels were 1.05±0.1 ng/ml for the control group and 1.34±0.07 ng/ml for the stress group.

When comparing the klotho and TNF-α serum levels, a statistically significant difference was determined to be present between the control and stress groups ($p<0.05$). A significant

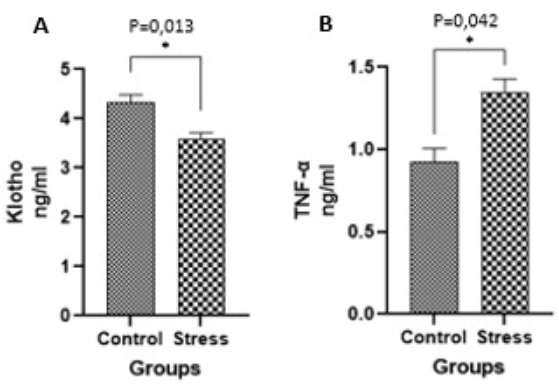

Figure 1: Relationship of klotho and TNF-α levels in serum between groups. A) Serum klotho (ng/ml) levels B) Serum TNF-α (ng/ml) levels.

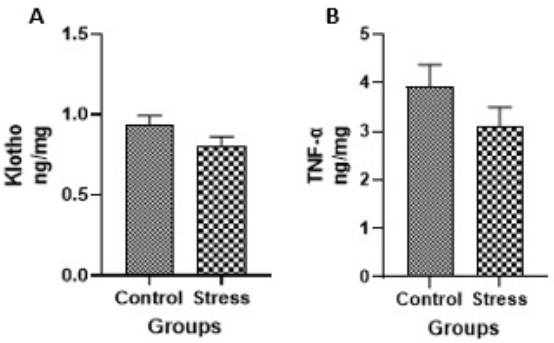

Figure 2: Mean klotho and TNF-α levels in liver tissue. A) Liver tissue klotho (ng/mg) levels B) Liver tissue TNF-α (ng/mg) levels.

decrease was observed in the stress group rats regarding klotho in the serum (p=0.013). When examining the TNF-α levels in the rats from the stress group, a significant increase was observed to have occurred (p=0.042). The stress factor was determined to have decreased the serum klotho level and to have increased the TNF-α level. Figure 1 shows the comparison of the klotho-TNFα levels between the two groups.

When considering the klotho and TNF-α levels in the liver tissue, no statistically significant difference was found between the control and stress groups (p>0.05). The mean klotho level in the liver tissue was 0.93±0.05 ng/mg for the control group and 0.80±0.05 ng/mg for the stress group. The mean TNF-α level in the liver tissue was 3.91±0.46 ng/mg for the control group and 3.10±0.38 ng/mg for the stress group. Figure 2 shows the mean klotho and TNF-α levels in the liver tissue for the two groups.

When comparing the klotho and TNF-α levels in the aortic tissue, no statistically significant difference was found between the control and stress groups (p>0.05). Furthermore, the mean TNF-α levels were 1.15±0.55 ng/mg for the control group and 2.65±0.83 ng/mg for the stress group. An evident increase in TNF-α levels had occurred in the aortic tissue, but this was not considered statistically significant (p=0.052). The mean klotho

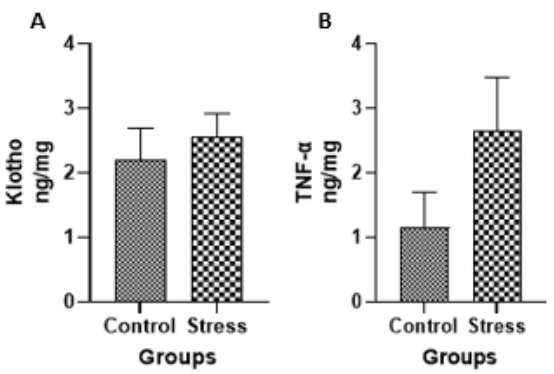

Figure 3: Mean klotho and TNF-α levels in aortic tissue. A) Aortic tissue klotho (ng/mg) levels B) Aortic tissue TNF-α (ng/mg) levels

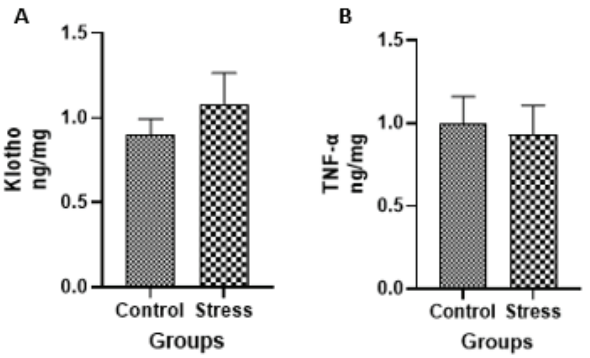

Figure 4: Mean klotho and TNF-α levels in heart tissue. A) Heart tissue klotho (ng/mg) levels B) Heart tissue TNF-α (ng/mg) levels.

level in the aortic tissue was 2.20±0.48 ng/mg for the control group and 2.56±0.35 ng/mg for the stress group. Figure 3 shows the mean klotho and TNF-α levels in the liver tissue for the two groups.

When comparing the klotho and TNF-α levels in the heart tissue, no statistically significant difference was found between the control and stress groups (p>0.05). The klotho levels in the heart tissue were 0.89±0.09 ng/mg for the control group and 1.08±0.18 ng/mg for the stress group. The mean TNF-α levels in the heart tissue were 0.99±0.16 ng/mg for the control group and 0.93±0.17 ng/mg for the stress group (Figure 4).

DISCUSSION

Stress is a term with broad significance and is employed to depict the physiological and psychological responses that arise within an organism due to the influence of a stimulus (1). Furthermore, stress has been identified as a disturbance of adaptation. In response to stress, organisms unleash a defense mechanism and are roused into action to restore homeostasis (2). As such, Selye contended stress to be a physiological response that is set into motion when an organism encounters a stimulus that challenges its ability to adapt (7).

In the initial stages of the stress response, the intermedia lateral region of the thoracolumbar spinal cord experiences an upsurge in activity in the preganglionic sympathetic neurons. The activation of these cells is transmitted to the paravertebral ganglia and subsequently to the chromaffin cells of the adrenal medulla (6). This stimulation brings about the fight-or-flight response, which was initially identified by Walter Cannon (1). Along with the biological damage and disorders that stress causes in tissues and organs, ischemia may also occur (25). The cardiovascular system and metabolic activity are the first biological systems to suffer in the face of stress (5).

Chronic stress-induced changes in an organism typically contribute to an increase in pro-inflammatory cytokines, including TNF-α (3). Several studies have suggested a correlation between stress and changes in the number of circulating T, B, and NK

cells. Evidence derived from both animal models and clinical studies supports the association between inflammation and depression. Exposure to inflammatory cytokines like TNFα or the use of cytokine inducers such as LPS has demonstrated significant behavioral changes in both human subjects and rats (8). Cross-sectional investigations have revealed associations between psychosocial factors, including low socioeconomic status, chronic job stress, caregiver tension, early-life adversity, hostility, and social isolation, as well as the circulating levels of TNF-α, C-reactive protein, and interleukin-6. Although the pathophysiology of the relationship between stress and immunology has not been fully elucidated, a clear association between the two has been established. TNF-α can contribute to increased neuroinflammation in the brain, potentially leading to impairment in nerve cells and connections. This can disrupt neuronal functions, consequently causing a decline in cognitive abilities such as memory loss. This effect may negatively impact neuronal health by enhancing the immune response in nerve cells, alongside other inflammatory factors such as NF-κB, COX-2, and IL-1 (4-8-26). A study conducted on medical students collected blood samples several weeks before, one day before, and immediately after their exams to investigate changes in proinflammatory cytokines. The study demonstrated psychological stress to be able to affect the production of proinflammatory and immune-regulatory cytokines. Students exhibiting high anxiety responses displayed markedly elevated production levels of TNF-α, IL-6, IL-1 receptor antagonist (IL-1Ra), interferon-gamma (IFN-gamma), and IL-10 in comparison to their counterparts without anxiety (3). Patients diagnosed with major depression have exhibited increases in the concentrations of inflammatory mediators such as chemokines, adhesion molecules, and prostaglandins in peripheral blood compared to controls, indicating the manifestation of all main features of inflammation (26). The similarity between the behavioral symptoms induced by cytokines and depression is striking (4). In both conditions, a physical and social withdrawal occurs accompanied by pain, fatigue, and decreased reactivity to reward (anhedonia). The severity of symptoms in both conditions is associated with increases in peripheral blood cytokine concentrations (9-27).

Stress factors are primarily perceived as neurobiological elements (28). When exposed to stressors, the brain primarily stimulates the synthesis of neuroendocrine hormones in the HPA axis (2). Differences arise in the biological pathway alongside the chronicity of the stressor (6). Chronic stress induces heightened synthesis of the high mobility group box-1 (HMGB-1) protein in microglial cells, mirroring conditions akin to ischemia and injury. Consequently, HMGB-1 amplifies the expression of TLR2-4 in immune cells, initiating a cascade of biological responses that prompt chemotactic cell mobilization and the secretion of TNF-α (29). The relationship between stress and inflammation is noteworthy, even in the absence of a microbial agent, as the immune response observed is quite remarkable. The current study shows similar results. When comparing the stress group with the control group, a significant increase in serum TNF-α levels was detected in the stressed group. However, no similar increase was observed regarding TNF-α levels in the tissue. No significant difference was detected in the heart, aorta, or liver tissues between the groups.

Klotho is a polypeptide with important roles in regulating the blood electrolyte balance (10). Klotho is defined as a co-activator of the cellular receptor FGF23 and is closely related to cellular aging (15). Studies have shown transgenic rats with an overexpression of klotho to have significantly longer lifespans, while those subjected to gene silencing experiments have shorter lifespans accompanied by many metabolic defects (10). Studies involving this interesting hormone-like protein have also shown the factors that support healthy living to increase klotho levels. For example, regular exercise, sleep, and a balanced diet have been reported to increase klotho levels (13-30). Conversely, klotho levels decrease in situations such as smoking, alcohol consumption, major depression, and obesity compared to control groups (17). One study investigating caregivers of children with autism and typically developing children reported psychosocial stress to reduce klotho levels, with the serum klotho levels of caregivers of autistic children with high-stress symptoms being significantly lower than those of the other caregiver group (16). A new in vivo study showing the relationship between psychological stress and klotho revealed klotho expression to be genetically increased and decreased in the nucleus accumbens (NAc), a brain region that regulates behavioral mood. When silencing klotho expression in mice, they exhibited major depressive behavior patterns, while an antidepressant effect was stated to occur in mice whose klotho expression had been increased (31). Drawing upon the current research findings, this study purports to lend credence to the existing literature. Remarkably, the study witnessed a striking reduction in klotho levels within the serums of rats that had been exposed to CUMs. In contrast to the control group, the klotho levels in the stress group exhibited a significant drop in their serum, despite no marked difference being discerned in their tissue klotho levels.

Klotho emerges as a newly identified hormone with high therapeutic potential (11). It has been linked to longevity, and its independent effect against stress provides a wide field of study (16, 17). Klotho protects against oxidative stress factors in an organism (20). The klotho protein has recently been described as a hormone that inhibits insulin/insulin-like growth factor-1 (IGF-1) signaling (11). The klotho protein activates the forkhead Box O (FOXO) transcription factors that are negatively regulated by the insulin/IGF-1 signal and induces the expression of manganese superoxide dismutase. This facilitates the removal of reactive oxygen species and provides resistance to oxidative stress. Therefore, inhibition of the insulin/IGF-1 signaling potentially contributes to klotho's anti-aging properties and is associated with increased resistance to oxidative stress (18). Another study observed a significant decrease in klotho expression in the RT-PCR analysis of cell lines that had been exposed to oxidative stress. Apoptosis has also been reported to be significantly reduced in cells experimentally overexpressing klotho (20). Proinflammatory cytokines increase the expressi-

on of adhesion proteins in cells. The klotho protein was exogenously applied to cell lines through the induction of TNF-α and reduced the expression of adhesion molecules (ICAM-1, VCAM-1). Klotho has been shown to activate a biological step toward reducing inflammation activity (19). Upon evaluating TNF-α and klotho levels together, this study observed that, while klotho levels had decreased in the stressed group, TNF-α levels had increased. The negative correlation between klotho and TNF-α is consistent with the literature. CUMS reduced the serum klotho level, which is associated with longevity. In contrast, the stress group showed a significant increase of the proinflammatory factor TNF-α levels in the serum.

Limitations of the study

The study used the ELISA method to measure TNF and klotho levels in the serum and heart, aorta, and liver tissues. In this way, the study determined the level differences in serum and tissues. However, the study's limitation lies in the absence of any histological assessment using the immunohistochemical method.

CONCLUSION

TNF-α is one of the main cytokines of inflammation, and this study observed a significant increase in TNF-α and a decrease in the klotho hormone to occur under chronic stress. The serum samples obtained from the stress group were found to have a significant decrease in klotho levels when TNF-α levels were high. However, no significant difference was found to have occurred in the TNF-α and klotho levels in the heart, aorta, and liver tissues among the groups.

As a result, TNF-α and klotho levels were found to be related to the changes caused by CUMS. By comparing the groups, the study established a relationship between the changes in serum and tissue. The study holds the conviction that the acquired data possess the potential to enrich the existing literature concerning the interconnections among stress, inflammation, and longevity.

Ethics Committee Approval: This study was approved by Animal Experiments Local Ethics Committee, Kocaeli University (Date: 29.03.2023, Approval No: 3/4-2023).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- M.D.Y., T.Ç.; Data Acquisition- M.D.Y., E.A.; Data Analysis/Interpretation- M.D.Y., E.A.; Drafting Manuscript- M.D.Y., T.Ç., E.A.; Critical Revision of Manuscript- M.D.Y., T.Ç., E.A., F.C.E.; Final Approval and Accountability- M.D.Y., T.Ç., E.A., F.C.E.; Material and Technical Support- F.C.E., E.A.; Supervision- T.Ç., F.C.E.

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