Original Article

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Heat Stress Alters Oxidative and Inflammatory Responses in Many Tissues of Male Rats

Sıcaklık Stresi, Erkek Sıçanların Birçok Dokusunda Oksidatif ve Enflamatuvar Yanıtları Değiştirir

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ABSTRACT

Objective: High ambient temperature beyond the comfort zone causes environmental heat stress (HS). A large number of free radicals are produced as a response to stress. This situation can cause morbidity and mortality in humans and animals. The current study was conducted to determine the effects of HS on antioxidant status, immune response and related factors.

Methods: In the study, 18 Sprague Dawley male rats were used as animal materials. Rats were randomly assigned to Thermo-neutral group (TN; untreated at normal ambient temperature of 24±2 °C), Heat-stress 1 group (HS1; 8 hours daily exposure at 30 °C) and Heat-stress 2 group (HS2; 8 hours daily exposure at 35 °C). At the end of HS application (day 14), rats were sacrificed under ether anesthesia and brain, duodenum, heart, liver, thyroid and testis tissues were taken. The biochemical and inflammatory responses of the tissues were analyzed with the ELISA Kit.

Results: Compared to the control group; Myeloperoxidase activity was higher in the heart tissue of HS2 group ($p<0.05$). Superoxide dismutaz activity was higher in the brain and testis tissues in HS2 group ($p<0.05$). Interleukin (IL)-2 level was higher in the duodenum and testis tissue of HS1 group and lower in the liver tissue ($p<0.05$). IL-6 level was higher in the brain and heart tissue of HS2 group ($p<0.05$). Tumor necrosis factor-α concentration was lower in the brain tissue in HS1 group, higher in the duodenum tissue in HS2 group and testicular tissue in HS1 group ($p<0.05$).

Conclusion: These results showed that HS adversely affected the oxidant/antioxidant status of brain, heart and testis tissues of rats and immune response of brain, duodenum, heart, liver and testis

ÖZ

Amaç: Konfor bölgesinin ötesindeki yüksek ortam sıcaklığı, çevresel sıcaklık stresine (HS) neden olur. Strese tepki olarak çok sayıda serbest radikal üretilir. Bu durum insanlarda ve hayvanlarda morbidite ve mortaliteye neden olabilir. Mevcut çalışma, HS'nin antioksidan durumu, bağışıklık tepkisi ve ilgili faktörler üzerindeki etkilerini belirlemek için yapılmıştır.

Yöntemler: Çalışmada hayvan materyali olarak 18 adet Sprague Dawley erkek sıçan kullanıldı. Sıçanlar rastgele olarak Termo-nötr grup (TN; HS uygulanmamış ve 24±2 °C normal ortam sıcaklığında işlem görmemiş), HS1 (HS1; 30 °C'de 14 gün boyunca günde 8 saat maruziyet) ve HS2 grubu (HS2; 35 °C'de 14 gün boyunca günde 8 saat maruziyet) olmak üzere 3 gruba ayrıldı. HS uygulaması sonunda (14. gün) eter anestezisi altında sıçanlar sakrifiye edildi ve beyin, duodenum, kalp, karaciğer, tiroid ve testis dokuları alındı. Dokuların biyokimyasal ve enflamatuvar yanıtları ELISA Kit ile analiz edildi.

Bulgular: Kontrol grubu ile karşılaştırıldığında; HS2 grubunun kalp dokusunda miyeloperoksidaz aktivitesi yüksekti ($p<0,05$). HS2 grubunda beyin ve testis dokularında süperoksit dismutaz aktivitesi yüksekti ($p<0,05$). İnterlökin (IL)-2 düzeyi HS1 grubunun duodenum ve testis dokusunda yüksek, karaciğer dokusunda düşüktü ($p<0,05$). HS2 grubunun beyin ve kalp dokusunda IL-6 düzeyi yüksekti ($p<0,05$). Tümör nektroz faktör-α konsantrasyonu HS1 grubunda beyin dokusunda düşük, HS2 grubunda duodenum dokusunda yüksek ve HS1 grubunda testis dokusunda yüksek bulundu ($p<0,05$).

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ABSTRACT

tissues; also showed that the negative effects of exposure to HS were related to the severity of the stress in the heart, liver and testicular tissues.

Keywords: Immune function, climate change, oxidative stress, heat stress

ÖZ

Sonuç: Bu sonuçlar, HS'nin sıçanların beyin, kalp ve testis dokularının oksidan/antioksidan durumunu ve beyin, duodenum, kalp, karaciğer ve testis dokularının bağışıklık tepkisini olumsuz etkilediğini; ayrıca HS'ye maruz kalmanın olumsuz etkilerinin kalp, karaciğer ve testis dokularında stresin şiddeti ile de ilişkili olduğunu göstermiştir.

Anahtar Sözcükler: Bağışıklık fonksiyonu, iklim değişikliği, oksidatif stres, sıcaklık stresi

Introduction

Stress is defined as any internal or external stimulus that produces a biological response. The impact of stress varies based on the type, timing, and severity of the stimulus applied. It can result in anything from alterations in homeostasis to life-threatening consequences, and in extreme cases, death (1). The stress response is a multifaceted complex mechanism that depends on many factors such as environmental variables (ambient temperature, humidity, solar radiation and air movement) and living factors (species, breed, age, health and physiological status) (2-4).

The range of environmental temperatures in which organisms comfortably perform their regular physiological activities is termed the "thermal neutral zone" or "comfort zone". One of the primary determinants of this zone is air temperature. Disruptions occur when the ambient temperature surpasses 25°C, disrupting the equilibrium between body temperature and body heat release. This condition, known as heat stress (HS), poses risks to human and animal health, leading to substantial economic losses in public health services and livestock production (5-7).

Numerous studies report that HS causes endocrine system disorders, electrolyte imbalance, immune system disorders, and lipid and protein oxidation (8-10). Additionally, HS is associated with oxidative stress (OS), marked by an upsurge in the production of reactive oxygen species (ROS) and a decline in antioxidant capacity (11,12). OS arises as a result of an imbalance between ROS production and the antioxidant defense against them. OS, which can cause damage to biomacromolecules such as DNA, proteins and lipids, can also result in cell dysfunction and tissue damage. OS induced by free radicals can cause many diseases such as cancer, Alzheimer's disease and autoimmune diseases as well as leading to degenerative processes (12-14).

Tissue myeloperoxidase (MPO), a marker of neutrophil migration, plays an important role in oxidant production by neutrophils. MPO enzyme is a lysosomal enzyme secreted from leukocytes in response to active stress. In the event of inflammation, MPO is released into the extracellular environment. Therefore, measurement of MPO activity as an indicator of neutrophil secretion is a sensitive test (15). Superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation of oxygen and hydrogen peroxide and is a primary protector against oxyradicals. SOD is an important antioxidant for superoxide, one of the main ROS in the cell (16). HS can stimulate systemic release of endogenous molecules and trigger local and systemic release of both pro- and anti-inflammatory cytokines such as interleukin (IL)-1 beta, IL-2, IL-6 and tumor necrosis factor (TNF)-α (17).

HS acts as the major challenge for maintaining the immune status in living organism. Acute HS may have a stimulatory effect on the immune system, however chronic HS may have an inhibitory role on the capacity of the immune system to maintain homeostasis (18). In addition, the negative impact of HS is predicted to worsen with the global warming (19).

This study aims to elucidate the effects of elevated temperature stress, specifically at 30°C and 35°C, on OS and inflammation biomarkers across different tissues. The selected parameters for this study are geared towards assessing inflammation and OS. TNF-α, IL-2, and IL-6 were chosen to probe inflammatory pathways, with MPO activity reflecting pro-oxidant processes, and SOD representing the enzymatic component of antioxidant activities.

Methods

In this study, 18 male Sprague Dawley rats from Atatürk University Experimental Animal Research Center were used. The Atatürk University local ethic committee for animal experiments approved the study, with protocol number: 2019-16/238.

Animals and Experimental Design

Eighteen Sprague Dawley male rats weighing between 250-300 g were randomly divided into three groups with six rats in each group. The groups were determined as Thermo-neutral (TN), 30 °C HS1 and 35 °C HS2.

Thermo-neutral (TN) group: The rats in this group were housed at room temperature at 22-24 °C for 24 hours/day and were fed with a normal diet and standard conditions were applied.

Temperature stress (HS1) group: The rats in this group were exposed to a temperature of 30 °C for 8 hours (20) a day (9:00 to 17:00/whole study) in wire cages in temperature-controlled rooms for fourteen days.

Temperature stress (HS2) group: The rats in this group were exposed to a temperature of 35 °C for 8 hours a day (from 9:00 to 17:00/whole study) in wire cages in temperature-controlled rooms for fourteen days.

At the end of the study, our experimental animals in the groups were stopped from being fed, as they were free to drink water from 24:00 the day before they were sacrificed by cervical dislocation method. Brain, duodenum, liver, thyroid gland and testis tissues of rats were taken for biochemical and histopathological analysis and stored at -80.

Analysis of Oxidative Stress (MPO) and Antioxidant Enzymes (SOD) in Various Tissues

Biochemical kits were used for measuring: Myeloperoxidase; Rat MPO ELISA kit, (Sunred biological technology; Shangai, China) and Superoxide dismutaz; Rat SOD ELISA kit, (Sunred biological technology; Shangai, China)

Determination of Inflammatory Markers (TNF-α, IL-2, IL-6) in Brain, Duodenum, Heart, Liver, Thyroid and Testis Homogenate of Adult Male Rat

Serum cytokines; IL-2, IL-6 and TNF-α, were measured using a commercial available kits specific for rats (ELISA kits, Sunred biological technology; Shangai, China). Levels of IL-2 and TNF-α in serum samples were measured according to manufacturer's instructions.

Statistical Analysis

Descriptive statistics of quantitative variables in the groups were expressed as mean and standard error. The Shapiro-Wilk test was used to confirm the normality of the distributions of quantitative variables. The homogeneity of variances, which was a prerequisite for parametric tests, was checked with Levene's test. Differences between groups within the same parameter were checked with one-way ANOVA and Kruskal-Wallis test. Tukey HSD and Dunnett's post-hoc tests were used to determine the groups caused the significant difference. Groups with significant differences within the same parameter were indicated with different superscript letters. P<0.05 was considered significant in all statistical calculations. SPSS (version 20.0, SPSS Inc, Chicago) package program was used for statistical analysis of the data.

Results

In this study, 18 male Sprague Dawley rats were used. The experimental animals were divided into three groups. The groups were exposed to a comfort temperature, 30 °C, and 35 °C, respectively. At the end of the experimental procedure, inflammatory markers (IL-2, IL-6, and TNF-α) in the brain, duodenum, heart, liver, thyroid, and testis tissues; MPO as an oxidative stress marker; and SOD levels indicating antioxidant activity were determined. (Tables 1-3 and Figure 1).

Biochemical Results

Brain

As shown in Table 1 and Figure 1, heat exposure in the brain tissue resulted in a higher IL-6 level and SOD activity, and a lower TNF-α concentration in the HS2 group compared to the control group (p<0.05, Table 1 and Figure 1).

Duodenum

Compared to the control group, HS1 group duodenal IL-2 level was higher in rats exposed to heat, and HS2 group duodenal TNF-α concentration was higher compared to the control group as well (p<0.05, Table 1 and Figure 1).

Heart

As shown in Table 2 and Figure 1, IL-6 and MPO levels of the heart tissue of the HS2 group exposed to 35 °C temperature were higher than the control and HS1 groups (p<0.05).

Liver

Heat exposure in the liver resulted in decreased IL-2 levels compared to the control group (p<0.05). Hepatic IL-6 level increased in the HS2 group compared to the HS1 group (p<0.05, Table 2 and Figure 1).

Table 1. Effects of Heat Stress Exposure on Oxidant/Antioxidant and Inflammatory Biomarkers in Brain and Duodenal Tissues.

	Control	HS1 (30 °C)	HS2 (35 °C)	*p
Parameters **Brain**	Mean ± SEM	Mean ± SEM	Mean ± SEM	
IL-2	.2282±.0466	.2262±.0511	.2697±.0477	.778
TNF- α	.4694^{a}±.0250	.3794^{b} ±.0126	.4491^{ab}±.0170	**.014**
IL-6	.1853^{a}±.0175	.2348^{ab}±.0412	.3136^{b}±.0221	**.026**
MPO	.2568±.0314	.2351±.0337	.1906±.0044	.368
SOD	.1754^{a}±.0188	.2134^{ab}±.0406	.3044^{b}±.0424	**.045**
Duodenal				
IL-2	.1421^{a}±.0081	.2614^{b}±.0627	.1506^{ab}±.0135	**.044**
TNF- α	.1498^{a}±.0105	.2362^{ab}±.0396	.2603^{b}±.0346	**.042**
IL-6	.1897±.0115	.2432±.0432	.1899±.0220	.349
MPO	.2144±.0583	.2139±.0528	.2468±.0062	.763
SOD	.1443±.0138	.1514±.0089	.2122±.0360	.468

SEM: Standard error of mean, *p<0.05 is considered statistically significant. Bold texts are important; ^{a,b}: Means followed by different superscript letter in the same row are significantly different (p<0.05).
TNF: Tumor necrosis factor, MPO: Myeloperoxidase, SOD: Superoxide dismutase

Thyroid

As shown in Table 3 and Figure 1, no statistically significant change was observed in thyroid tissue IL-2, IL-6, TNF-α, MPO and SOD values at 30 °C and 35 °C exposure (p>0.05).

Testis

Exposure to heat stress in testicular tissue resulted in an elevation of IL-2 levels in the HS1 group compared to controls (p<0.05). Testicular concentrations of TNF-α were increased in both HS1 and HS2 groups relative to the control group (p<0.05), and SOD activity in testicular tissue also showed a marked increase compared to controls (p<0.05).

Discussion

HS is increasingly significant due to global warming (21,22). Exposure of the body to heat can cause different physio/pathological conditions. Behavioral and autonomic temperature-defense negative feedback mechanisms are used to maintain normal body temperature. Physiologically, successful activation of defense mechanisms results in temperature tolerance. With repeated, chronic heat exposure, tolerance intensifies, leading to heat acclimation. Heat-related diseases develop when the pathological effects of heat load are not prevented. Syndromes range from less severe such as heat syncope to severe forms such as fatal heatstroke (23) .

Table 2. Effect of heat stress exposure on liver and heart tissues oxidant/antioxidant and inflammatory biomarkers

	Control	HS1 (30 °C)	HS2 (35 °C)	*p
Liver	Mean ± SEM	Mean ± SEM	Mean ± SEM	
IL-2	.2881^{a}±.0046	.2116b±.0499	.2418^{ab}±.0044	.040
TNF- α	.4194±.0540	.4297±.0676	.3233±.0758	.481
IL-6	.2597^{ab}±.0457	.2116b±.0290	.3753^{a}±.0137	.027
MPO	.2395±.0387	.2225±.0456	.1506±.0205	.292
SOD	.1958±.0321	.1311±.0060	.1590±.0105	.068
Heart				
IL-2	.3942±.0105	.3050±.0570	.2440±.0642	.144
TNF- α	.5015±.0409	.4429±.0332	.3952±.0214	.113
IL-6	.2352^{a}±.0149	.3073^{a}±.0323	.3911^{b}±.0018	.004
MPO	.1900^{a}±.0046	.1799^{a} ±.0152	.3073^{b}±.0483	.017
SOD	.1807±.0133	.1400±.0124	.1934±.0258	.138

SEM: Standard error of mean, *p<0.05 is considered statistically significant. Bold texts are important; a,b: Means followed by different superscript letter in the same row are significantly different (p<0.05).
TNF: Tumor necrosis factor, MPO: Myeloperoxidase, SOD: Superoxide dismutase

Table 3. Effect of exposure to heat stress on oxidant/antioxidant and inflammatory biomarkers of thyroid and testicular tissue

	Control	HS1 (30 °C)	HS2 (35 °C)	*p
Parameters Thyroid gland	Mean ± SEM	Mean ± SEM	Mean ± SEM	
IL-2	.3038±.0687	.2093±.0529	.2422±.0673	.595
IL-6	.3312±.0700	.3327±.0577	.2889±.0319	.801
TNF- α	.3880±.1000	.3577±.0341	.2920±.0062	.619
MPO	.2911±.0710	.2870±.0482	.3392±.0033	.726
SOD	.1566±.0188	.1828±.0272	.2002±.0192	.441
Testicular tissue				
IL-2	.1729^{a} ±.0271	.2985^{b}±.0438	.2256^{ab}±.0159	**.045**
TNF- α	.2811^{a} ±.0152	.6000^{b}±.0303	.3512^{a}±.0340	.000
IL-6	.2073 ± .0251	.2929 ± .0375	.2955 ± .0082	.084
MPO	.2135±.0390	.2806±.0625	.3634±.0892	.309
SOD	.1276^{a} ±.0152	.1846^{ab} ±.0413	.2478^{b}±.0044	**.016**

SEM: Standard error of mean, *p<0.05 is considered statistically significant. Bold texts are important; ^{a,b}: Means followed by different superscript letter in the same row are significantly different (p<0.05)
TNF: Tumor necrosis factor, MPO: Myeloperoxidase, SOD: Superoxide dismutase

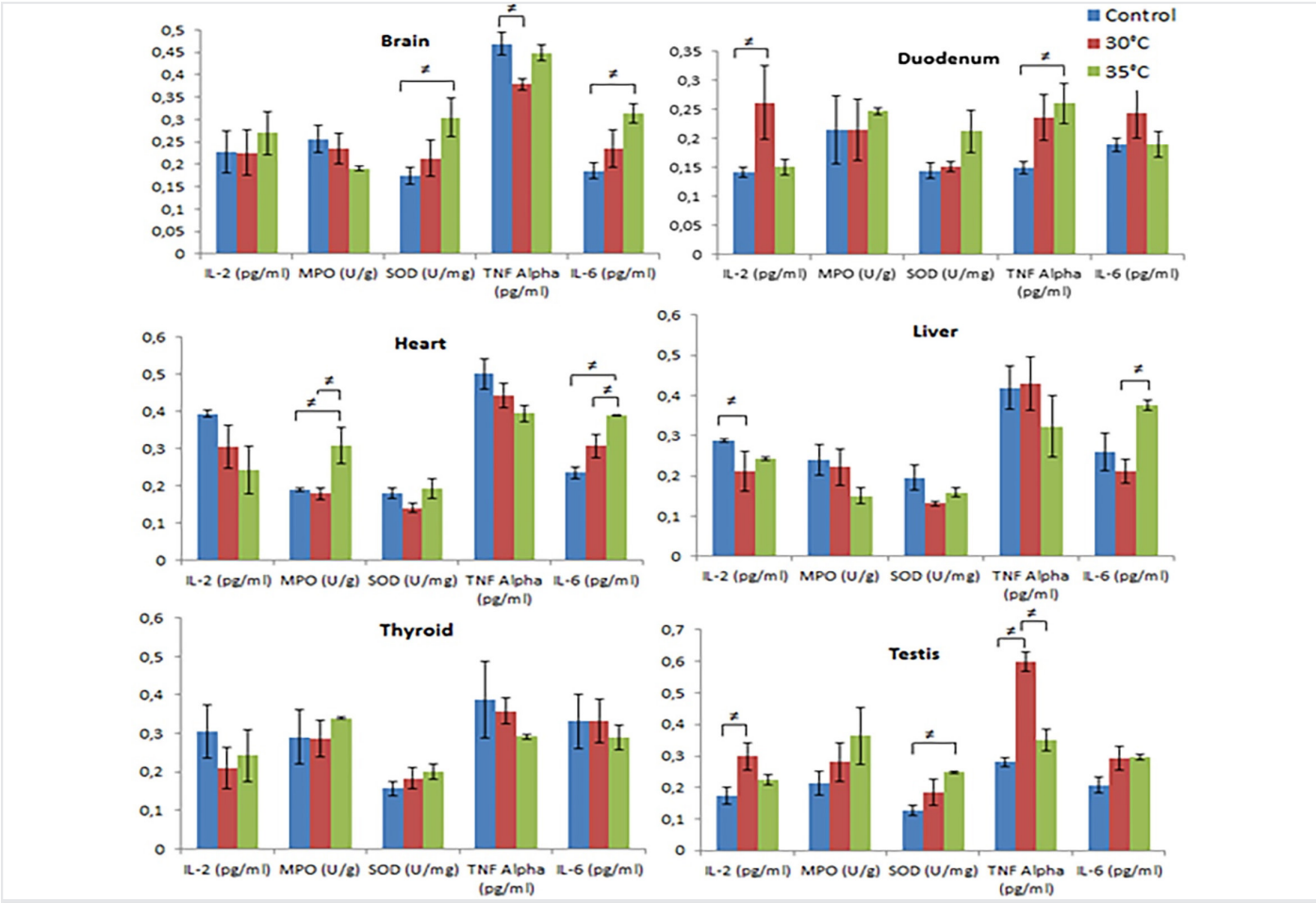

Figure 1. Effects of heat stress exposure on oxidant/antioxidant and inflammatory biomarkers across various tissues.

There are number of internal environment changes and systemic effects caused by HS in the organism as determined in the literature. However, our study determined the effects of high-temperature stress exposures, beyond the comfort temperature, on the oxidant system (MPO, SOD) and inflammation markers (IL-2, IL-6, TNF-α levels) in various tissues, supplementing existing literature (Tables 1-3 and Figure 1).

HS changes the physiology of all systems in the body with impaired redox homeostasis (24). MPO, secreted from activated polymorphonuclear leukocytes, increases OS by promoting free oxygen radical production.Therefore, excessive MPO production can damage tissues and organs (25). It was determined in our study that the heart tissue MPO level of the HS2 group was higher than that of the control group and HS1 group ($p<0.05$, Table 2 and Figure 1). This result shows that exposure to 35 °C HS increases the oxidant level associated with heart tissue leukocyte activation. In a study conducted by Horowitz et al. (26), the tissue damage caused by HS was associated with increased free radical production in animals exposed to high ambient temperature (42 or 43 °C). In another study, it was reported that chickens exposed to 36 °C for 8 hours a day showed a rapid increase in ROS production in mitochondria of duodenal, jejunal and ileal epithelial cells (27). An *in vitro* study with intestinal epithelial cells showed that exposure to 42 °C for 60 minutes increased ROS concentration and mitochondrial dysfunction and early apoptotic rates (28).

Free radicals produced continuously in the cell are destroyed by the antioxidant defense systems that are produced during normal metabolism in the body. Antioxidants basically prevent or delay cell damage by scavenging free radicals in the cell. Antioxidants can either be produced naturally in the body or obtained from external foods (29). SOD is the most important antioxidant defense system against ROS and superoxide anion radicals (30). When Table 1 and Table 3 were examined, it was seen that 35 °C temperature exposure increased the SOD activity of brain and testis tissues ($p<0.05$). The increase in the activity of SOD, an antioxidant enzyme, in brain and testicular tissues may be an antioxidant defense response to increased reactive oxygen intermediates.

Cytokines are intracellular peptides that act as immune system mediators. In both human and animal models, the levels of pro-inflammatory and anti-inflammatory cytokines increase in heat stroke (17). The inflammatory reaction associated with heatstroke includes activation of the humoral and cellular components of the immune system, as well as alterations in the production and/or suppression of numerous cytokines (31-

33). The findings of this study suggest that HS is associated with changes in proinflammatory cytokine levels. When the inflammatory responses of the tissues are examined; duodenum TNF-α concentration, heart and brain IL-6 levels increased, while brain TNF-α concentration and liver IL-2 levels decreased ($p< 0.05$). *Ex vivo* and *in vitro* studies characterizing the direct effects of heat have shown that it activates numerous pathways related to the physiological or cellular/biochemical axis (17,34). In a study by Helwig and Leon (35) using a mouse model of heat stroke, it was reported that the IL1 cytokine family played a role in the development of organ damage (spleen and liver). Another study showed that exposure to heat directly affected intestinal permeability and function The inflammatory response in animals exposed to mild to severe heat has the potential to impair nutrient absorption, gut health, and immunological status of the affected organism (34,36).

The potent inflammatory response to heatstroke increases after the end of HS and is intricately involved in both the damaging processes and the repair mechanisms activated during the healing phase. In survivors, the magnitude of this response decreases as time progresses, allowing a return to normal homeostasis. A stronger inflammatory response is often associated with poor prognosis and even death, indicating an imbalance in the immune system and causing a dysregulated inflammatory response (17). Consequently, HS can cause organ dysfunction associated with the accumulation of oxidants and the release of proinflammatory mediators. Future research should aim to elucidate the role of the immune inflammatory response in heat-stressed organisms. Nutritional and therapeutic interventions can prevent damage to organs due to HS and increase thermal tolerance to heat by reducing the accumulation of oxidants, but additional data are needed to support this theory.

Study Limitations

It is important to acknowledge the limitations of the current study. Because only male rats used in the experiments, these findings might not apply to female rats. Further studies that include different genders, ages, ete. are necessary. Future research should consider this difference.

Conclusion

As a result, exposure to high temperatures (30 °C or 35 °C) outside the room temperature of 24 °C, which is known as the comfort temperature, causes changes in the redox status of rats, in oxidant and antioxidant levels, and also in the levels of cytokines, known as immune system mediators. This indicates that exposure to high temperatures may lead to homeostasis changes in living organisms, resulting in deterioration of tissue and organ functions.

Ethics

Ethics Committee Approval: The approval to our study was granted by Atatürk University Local Ethical Committee for Animal Experiments with the decision number 7704040475-641.04-E.1900352247 (dated: December 31, 2019 and numbered: 2019/238).

Informed Consent: This study does not apply because it involves animal subjects.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: E.S., H.U., Design: E.S., H.U., Data Collection or Processing: E.S., H.U., Analysis or Interpretation: E.S., H.U., Literature Search: E.S., H.U., Writing: E.S., H.U.

Conflict of Interest: No conflict of interest was declared by the authors.

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