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Esculetin Attenuates Doxorubicin-Induced Kidney Damage By Reducing Heat Shock Proteins Expression Levels

Eskuletin, Isı Şoku Proteinlerinin Ekspresyon Düzeylerini Azaltarak Doksorubisinin Neden Olduğu Böbrek Hasarını Azaltıyor

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ABSTRACT

The effectiveness of Doxorobucin (DOX), a commonly used anti-cancer and immunosuppressive medication, is hindered by its potential for organ toxicity. Prolonged use of DOX is associated with severe hepatocellular toxicity. Esculetin (E) is a naturally occurring compound that belongs to the coumarin family. It possesses antibacterial, antioxidant, anti-inflammatory, and anti-diabetic characteristics. This study reveals fresh insights into the therapeutic impact of E on DOX-induced kidney cell damage. E demonstrates its remedial effects by modulating heat shock protein signaling pathways. In our research, we explored the impact of DOX and E on the expression of the 70 kDa HSP gene family, including *Hspa1a*, *Hspa4*, and *Hspa5*, which are small stress proteins in *Rattus norvegicus*. The rats were divided into a total of six groups: control group, DOX group (administered DOX 5 mg/kg/every other day for two weeks), E50 group (administered 50 mg/kg/day of esculetin intraperitoneally), E100 group (administered 100 mg/kg/day of esculetin intraperitoneally) and combined groups (DOX+E50 and DOX+E100) in which esculetin was administered together with DOX. Subsequently, kidney tissues were collected from rats, and cDNA libraries were generated at the conclusion of the application process. The Real-Time PCR method was employed using these libraries to detect HSP70 genes. Analyses conducted on *Hspa1a*, *Hspa4* and *Hspa5* expression revealed a statistically significant increase in the DOX group compared to the control group ($p < 0.0001$). Additionally, the combination of DOX and esculetin demonstrates a reduction in the increase caused by DOX alone ($p < 0.0001$). The study suggests that esculetin could serve as a potential protective agent for shielding kidney tissue from oxidative damage and apoptosis.

Keywords: Doxorobucin, Esculetin, Heat shock proteins, Nephrotoxicity, Oxidative stress.

ÖZ

Yaygın olarak kullanılan bir anti-kanser ve immünosupresif ilaç olan Doxorobucin'in (DOX) etkinliği, organ toksisitesi potansiyeli nedeniyle engellenmektedir. DOX'un uzun süreli kullanımı ciddi hepatoselüler toksisite ile ilişkilidir. Eskuletin (E), kumarin ailesine ait doğal olarak oluşan bir bileşiktir. Antibakteriyel, antioksidan, anti-enflamatuar ve anti-diyabetik özelliklere sahiptir. Bu çalışma, E'nin DOX kaynaklı böbrek hücresi hasarı üzerindeki terapötik etkisine dair yeni bilgiler ortaya koymaktadır. E, ısı şoku protein sinyal yolaklarını modüle ederek iyileştirici etkilerini göstermektedir. Araştırmamızda, DOX ve E'nin *Rattus norvegicus*'ta küçük stres proteinleri olan *Hspa1a*, *Hspa4* ve *Hspa5* dahil olmak üzere 70 kDa HSP gen ailesinin ekspresyonu üzerindeki etkisini araştırdık. Sıçanlar toplam altı gruba ayrılmıştır: kontrol grubu, DOX grubu (iki hafta boyunca 5 mg/kg/iki günde bir intraperitoneal DOX uygulanmıştır), E50 grubu (intraperitoneal 50 mg/kg/gün eskuletin uygulanmıştır), E100 grubu (intraperitoneal 100 mg/kg/gün eskuletin uygulanmıştır) ve eskuletinin DOX ile birlikte uygulandığı kombine gruplar (DOX+E50 ve DOX+E100). Daha sonra sıçanlardan böbrek dokuları toplandı ve uygulama sürecinin sonunda cDNA kütüphaneleri oluşturuldu. HSP70 genlerini tespit etmek için bu kütüphaneler kullanılarak Real-Time PCR yöntemi kullanıldı. *Hspa1a*, *Hspa4* ve *Hspa5* ekspresyonu üzerinde yapılan analizler, DOX grubunda kontrol grubuna kıyasla istatistiksel olarak anlamlı bir artış olduğunu ortaya koydu ($p < 0.0001$). Ek olarak, DOX ve eskuletin kombinasyonu, tek başına DOX'un neden olduğu artışta bir azalma olduğunu göstermektedir ($p < 0.0001$). Çalışma, eskuletin'in böbrek dokusunu oksidatif hasar ve apoptozdan korumak için potansiyel bir koruyucu ajan olarak hizmet edebileceğini göstermektedir.

Anahtar kelimeler: Doksorobusin, Eskuletin, Isı şok proteinleri, Nefrotoksisite, Oksidatif stres.

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Introduction

Proteins play a crucial role in nearly all cellular biological and genetic functions (Kırıcı et al., 2016). The proper functioning of proteins relies on the accurate folding of their three-dimensional conformation, a vital aspect in a prerequisite for their physiological role and the biological context (Ceylan and Erdogan, 2017). Unfortunately, the constant occurrence of protein misfolding and subsequent aggregation poses a threat to cells, as the majority of proteins are inherently unstable in their journey to attain their native tertiary state (Ceylan and Erdogan, 2017).

Heat shock proteins (HSPs), recognized for their role in maintaining organismal balance to adapt to stress and regulating cellular responses for protection against intracellular and extracellular challenges, stand out as highly conserved proteins throughout evolutionary history (Dubrez et al., 2020; Guo et al., 2023). Functioning as molecular chaperones, HSPs serve as molecular binders between the cell and membrane proteins, actively participating in processes such as protein secretion, assembly, maintenance of structural protein integrity, protein degradation, folding, and trafficking, facilitating their correct folding (Shan et al., 2020; Lang et al., 2021).

In the realm of proteostasis, HSPs contribute to immune cell functions and responses, playing crucial roles in angiogenesis, signal transduction, apoptosis, cell proliferation, and positioning them as potential targets in cancer therapy (Albakova et al., 2021). The protective mechanisms of HSPs encompass the repair of damaged proteins, and elimination of irreparable proteins, suppression of proinflammatory cytokines (Tukaj, 2020), folding of nascent polypeptides (Boopathy et al., 2022). In response to stress signals and injury, HSPs undergo significant synthesis and binding to proteins, stabilizing/translating RNA and influencing apoptosis, alleviating protein denaturation and misfolding (Hu et al., 2022). In essence, HSPs play a beneficial role in cellular recovery and repair, ensuring cell survival. The inducible HSPA1A belongs to the 70-kDa HSPA family, functioning primarily as a chaperone to modulate proteostasis. However, it is noteworthy that HSPA1A also plays a role as a cytokine in the regulation of inflammation, earning it the designation of a "chaperokine." (De Freitas et al., 2022). HSPA4 is serving as a co-chaperone alongside HSP70. With ubiquitous expression, HSPA4 has demonstrated its capability to prevent inflammation and apoptosis, provide protection against oxidative stress, and enhance overall survival (Shang et al., 2021). HSPA5, alternatively recognized as glucose-regulated protein-78 (Dos Santos et al., 2023), assumes critical functions in facilitating the assembly of proteins within the endoplasmic reticulum (ER), the formation of protein complexes, and the orchestration of the unfolded protein response (Rehati et al., 2023).

Isolated from cultures of *Streptomyces peucetius* var. Caesius and discovered in the 1950s, doxorubicin (DOX) belongs to the anthracycline family of antibiotics. Esteemed as one of the most crucial antitumor agents in clinical use (Rawat et al., 2021). DOX serves as a first-line treatment for various cancer types, encompassing hematological neoplasms and solid tumors (Patel et al., 2022). Despite being in use since the 1960s, the clinical application of DOX is restricted by its potential for toxicity, leading to heightened levels of mortality (Van der Zanden et al., 2021). Characterized lesions in DOX-induced toxicity encompass compensatory muscle hypertrophy (Nguyen et al., 2024) muscle edema, fragmentation or myofibril loss, fibrosis, cytoplasmic necrosis, vacuolization alterations in nuclear size (Boussada et al. 2022). Notably, the primary risk factor for the development of chronic heart failure is the total cumulative DOX dose (Tian et al., 2020). Esculetin (6,7-dihydroxychromen-2-one), a natural phenolic compound classified within the coumarins group, which belongs to the class of benzopyrone enriched in various plants such as *Aesculus turbinata* and *Sonchus grandifolius* etc. (Hassanein et al., 2020). This particular chemical, classified as a coumarin, is recognized for its ability to preserve the liver, a property that can be related to its antioxidant characteristics. Tien et al. (2011) conducted a research demonstrating that treatment with esculetin at doses of 100 and 500 mg/kg may effectively eliminate free radicals produced during lipid peroxidation by enhancing the activity of antioxidant enzymes, including catalase (CAT) and superoxide dismutase (SOD). In a separate investigation, Lee et al. (2002) discovered that esculetin effectively decreased oxidative stress in experimental rat liver lesions. This was demonstrated by a noticeable decrease in leukocyte infiltration, edema and liver cell necrosis.

In the current study, under therapeutic settings, we aimed to analyze the effect of esculetin on HSP genes expression levels in the kidney of rats after DOX administration.

Materials and Methods

Materials

Forty eight Sprague Dawley rats (9-10 weeks old, weighing 180 g ± 10 g, *Rattus norvegicus*) were procured from Atatürk University Medical Experimental Research and Application Center. The animals were housed under standard conditions (temperature: 22°C ± 1°C, humidity: 60%, and 12-12 h lighting cycle) for one week prior to experimentation. Random selection was employed to distribute the animals into six groups (Con, DOX, E50, E100, DOX+E50, DOX+E100, n=8 as per the experimental design. (Desai et al., 2013).

Control group: Rats received intraperitoneal injections of normal saline for 14 days.

DOX group: Rats were intraperitoneally injected with DOX (5 mg/kg/ every other day) for 14 days.

E50 group: Rats received intraperitoneal injections of esculetin (50 mg/kg/day) for 14 days.

E100 group: Rats were injected intraperitoneally with esculetin (100 mg/kg/day) for 14 days.

DOX+E50 group: Rats were intraperitoneally injected with DOX, and esculetin was administered 1 hour before the DOX treatment, following the regimen of the DOX and E50 groups.

DOX+E100 group: Rats were injected intraperitoneally with DOX, and esculetin was administered 1 hour before the DOX treatment, following the regimen of the DOX and E100 groups.

Animals were euthanized by cervical dislocation 24 hours after these procedures. Tissue samples were rapidly removed, washed in PBS, and immersed in liquid nitrogen for further experiments, then frozen and stored at -86°C. Animal experiments were conducted following the National Research Council's Guide for the Care and Use of Experimental Animals and were approved by the ‘’Local Ethics Committee of Atatürk University Animal Experiments’’ (Protocol No: 2021/4–123).

Methods

RNA isolation and gene expression analysis

Total RNA isolation from rat kidney tissues was conducted using a commercial kit (EcoPURE Total RNA Kit, EcoTech). The isolation procedure followed the manufacturer's recommended protocol. After isolation, RNA purity (A260/A280) and concentration were determined using a Nanodrop device (MultiscanGo, Thermo Scientific). The obtained RNAs were stored at -86°C for subsequent use in cDNA synthesis Yeşilkent and Ceylan, 2023). cDNA synthesis was carried out using a commercial kit (iScript™ Reverse Transcription Supermix for RTqPCR, BioRad). The synthesis process followed the manufacturer's protocol. After synthesis, the products were stored at -20°C for use in the qPCR step (Yeşilkent and Ceylan, 2023). Primers for the target genes (Table 1) were designed using the Primer3 database (http://bioinfo.ut.ee/primer3-0.4.0/), and their binding properties were verified using the BLAST module (https://blast.ncbi.nlm.nih.gov/) (Untergasser et al., 2012). qPCR was conducted using primers specific to the target genes, following the protocol: SYBR Green Super Mix, Forward/Reverse Primer, ddH2O, cDNA components (SsoAdvanced Universal SYBR Green Supermix, BioRad). The ΔCt method was employed to assess the Ct values obtained (Toraman et al., 2022).

Table 1. Primer Sequence of studied genes

Tablo 1. Çalışılan genlerin primer dizisi

Gene Symbols		Sequence (5'-3')
Hspa1a	forward	ACGCTGGCTGAGAAAGAGG
	reverse	ATCCACCTCCTCGATGGTG
Hspa4	forward	TGCTTGGAAAGGCTGAGTGG
	reverse	TGGGAATAGAGGGCAATGAG
Hspa5	forward	ATTCCTGCGTCGGTGTATTC
	reverse	TGGACGTGAGTTGGTTCTTG

Statistical Analysis

The determination of the number of groups and the allocation of rats within each group were established through a thorough G-power analysis. G-power Software 3.1.9.7 (University of Dusseldorf, Germany) was employed for conducting the a priori power analysis, aiming to determine the minimum sample size required for the study. The analysis utilized F tests aligned with the study's design, which involved repeated measures ANOVA with both within and between factors analysis. The study comprised six groups with measurements taken at a single time point. The significance level (α) was set at 0.05, the minimum effect size was specified as 0.25, the correlation among

repeated measures was set at 0.5, and the nonsphericity correction was fixed at 1. To achieve a power (1-β error probability) of 0.80, the calculated minimum sample size necessary for statistical significance was determined to be 36 subjects in total, resulting in an actual power of 81.0%.

The normality of numerical variables was assessed utilizing the Shapiro-Wilk test. Results indicated that all variables exhibited a normal distribution ($p > 0.05$). All measurements were conducted in triplicates for each animal and sample. Statistical comparisons of the results were performed using one-way ANOVA followed by Tukey's post-hoc test with Prism software (GraphPad Software, San Diego, CA). Statistically significant differences are indicated as follows: $p > 0.05$ (indicating lack of significance), *:$p < 0.05$ (indicating significance), **:$p < 0.01$, ***:$p < 0.001$ and ****:$p < 0.0001$ (Toraman et al., 2022).

Results

In this study, 5 mg of DOX and 50-100 mg doses of E were administered to rats to evaluate the effects of DOX and E. Analyses conducted on *Hspa1a* expression revealed a statistically significant increase in the DOX group compared to the control group ($p < 0.0001$). On the other hand, E-only treatment did not show a significant change compared to the control at both doses (50-100 mg/kg/day). In the groups where the combination of DOX and E was evaluated, a noticeable decrease in *Hspa1a* expression was observed in the DOX+E50 and DOX+E100 forms compared to the DOX group ($p < 0.0001$). These findings indicate that DOX increases *Hspa1a* expression, and the application of E does not alter this effect compared to the control. Additionally, the combination of DOX and E demonstrates a reduction in the increase caused by DOX alone (Table 2,3 and Figure 1).

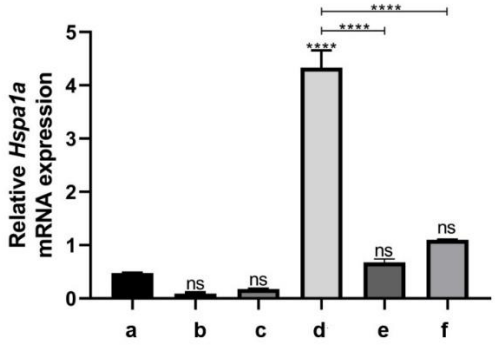

Figure 1. Analyses of the relative gene expression for *Hspa1a1* from the kidney tissues treated with DOX and E. Each bar represents the mean ± SEM. Asterisk (*) indicates statistically significant difference between the means (a: Control, b: DOX, c: E50, d:E100, e: DOX+E50 f: DOX+E100 ns:$p > 0.05$, *:$p < 0.05$, **:$p < 0.01$, ***:$p < 0.001$ and ****:$p < 0.0001$).

Şekil 1. DOX ve E ile tedavi edilen böbrek dokularında *Hspa1a1* için gen ekspresyon analizi. Her çubuk ortalama ± SEM'i göstermektedir. Yıldız işaretleri (*) ortalamalar arasındaki istatistiksel anlamlılığı göstermektedir (a: Kontrol, b: DOX, c: E50, d:E100, e: DOX+E50 f: DOX+E100 ns:$p > 0.05$, *:$p < 0.05$, **:$p < 0.01$, ***:$p < 0.001$ and ****:$p < 0.0001$)

Table 2. One-way ANOVA test results of *Hspa1a* qPCR data

Tablo 2. *Hspa1a* qPCR verilerinin tek yönlü ANOVA testi sonuçları

Groups	N	Mean	Std. Error	F	p
Control[a]	8	,47	,01		
E50[b]	8	,09	,00		
E100[c]	8	,17	,00	3116,971	,000*
DOX[d]	8	4,26	,06		
DOX+E50[e]	8	,66	,01		
DOX+E100[f]	8	1,06	,02		

*significant statistical differences were identified when comparing the groups based on DOX-E kidney *Hspa1a* mRNA levels (p≤ .000).

Table 3. Results of Tukey HSD test of *Hspa1a* qPCR data

Tablo 3. *Hspa1a* qPCR verilerinin Tukey HSD testi sonuçları

Tukey's multiple comparisons test	Significant
a vs. b	ns
a vs. c	ns
a vs. d	****
a vs. e	ns
a vs. f	*
b vs. c	ns
b vs. d	****
b vs. e	*
b vs. f	***
c vs. d	****
c vs. e	*
c vs. f	**
d vs. e	****
d vs. f	****
e vs. f	ns

a: Control, b: DOX, c: E50, d:E100, e: DOX+E50 f: DOX+E100, ns:p >0.05, *:p < 0.05, **:p < 0.01, ***:p < 0.001 and ****:p < 0.0001

Analyses conducted on *Hspa4* expression have shown a statistically significant increase in the DOX group compared to the control group ($p < 0.0001$). On the other hand, in the groups where E was applied, a statistically significant decrease in both doses compared to the control has been observed ($p < 0.001$). In the groups where the combination of DOX and E was evaluated, a noticeable decrease in *Hspa4* expression was observed in the DOX+E50 and DOX+E100 forms compared to the DOX group ($p < 0.0001$). The DOX+E50 group showed no substantial variation when compared to the control group, whereas a statistically significant decline was noted in the DOX+E100 group in comparison to the control group ($p < 0.01$). These findings indicate that DOX increases *Hspa4* expression (Table 4, 5 and Figure 2).

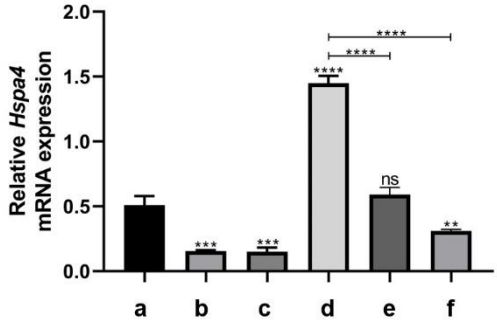

Figure 2. Analyses of the relative gene expression for *Hspa4* from the kidney tissues treated with DOX and E. Each bar represents the mean ± SEM. Asterisk (*) indicates statistically significant difference between the means (a: Control, b: DOX, c: E50, d:E100, e: DOX+E50 f: DOX+E100 ns:p >0.05, *:p < 0.05, **:p < 0.01, ***:p < 0.001 and ****:p < 0.0001).

Şekil 2. DOX ve E ile tedavi edilen böbrek dokularında *Hspa4* için gen ekspresyon analizi. Her çubuk ortalama ± SEM'i göstermektedir. Yıldız işaretleri (*) ortalamalar arasındaki istatistiksel anlamlılığı göstermektedir (a: Kontrol, b: DOX, c: E50, d:E100, e: DOX+E50 f: DOX+E100 ns:p >0.05, *:p < 0.05, **:p < 0.01, ***:p < 0.001 and ****:p < 0.0001).

Table 4. One-way ANOVA test results of *Hspa4* qPCR data

Tablo 4. *Hspa4* qPCR verilerinin tek yönlü ANOVA testi sonuçları

Groups	N	Mean	Std. Error	F	p
Control[a]	8	,52	,01		
E50[b]	8	,16	,00		
E100[c]	8	,15	,00		
DOX[d]	8	1,42	,01	1360,012	,000*
DOX+E50[e]	8	,58	,01		
DOX+E100[f]	8	,32	,00		

*significant statistical differences were identified when comparing the groups based on DOX-E kidney *Hspa4* mRNA levels ($p \leq .000$).

Table 5. Results of Tukey HSD test of *Hspa4* qPCR data

Tablo 5. *Hspa4* qPCR verilerinin Tukey HSD testi sonuçları

Tukey's multiple comparisons test	Significant
a vs. b	***
a vs. c	***
a vs. d	****
a vs. e	ns
a vs. f	**
b vs. c	ns
b vs. d	****
b vs. e	***
b vs. f	*
c vs. d	****
c vs. e	***
c vs. f	*
d vs. e	****
d vs. f	****
e vs. f	**

a: Control, b: DOX, c: E50, d:E100, e: DOX+E50 f: DOX+E100, ns:p >0.05, *:p < 0.05, **:p < 0.01, ***:p < 0.001 and ****: p < 0.0001

Analyses conducted on *Hspa5* expression have shown a statistically significant increase in the DOX group compared to the control group ($p < 0.0001$). On the other hand, in the groups where E was applied, there was no significant change in the E50 dose compared to the control, while a noticeable increase was observed in the E100 dose ($p < 0.01$). In the combination groups, a significant decrease in *Hspa5* expression was observed compared to the DOX group ($p < 0.0001$). The DOX+E50 group did not exhibit a

noteworthy alteration when compared to the control group. In contrast, a statistically significant rise was noted in the DOX+E100 group in comparison to the control group ($p < 0.001$, Table 6,7, Figure 3).

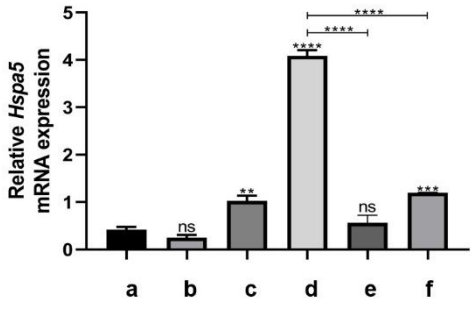

Figure 3. Analyses of the relative gene expression for *Hspa5* from the kidney tissues treated with DOX and E. Each bar represents the mean ± SEM. Asterisk (*) indicates statistically significant difference between the means (a: Control, b: DOX, c: E50, d:E100, e: DOX+E50 f: DOX+E100 ns:p >0.05, *:p < 0.05, **:p < 0.01, ***:p < 0.001 and ****:p < 0.0001).

Şekil 3. DOX ve E ile tedavi edilen böbrek dokularında *Hspa5* için gen ekspresyon analizi. Her çubuk ortalama ± SEM'i göstermektedir. Yıldız işaretleri (*) ortalamalar arasındaki istatistiksel anlamlılığı göstermektedir (a: Kontrol, b: DOX, c: E50, d:E100, e: DOX+E50 f: DOX+E100 ns:p >0.05, *:p < 0.05, **:p < 0.01, ***:p < 0.001 and ****:p < 0.0001).

Table 6. One-way ANOVA test results of *Hspa5* qPCR data

Tablo 6. *Hspa5* qPCR verilerinin tek yönlü ANOVA testi sonuçları

Groups	N	Mean	Std. Error	F	p
Control[a]	8	,41	,00		
E50[b]	8	,26	,01		
E100[c]	8	1,03	,02		
DOX[d]	8	4,06	,02	5356,226	,000*
DOX+E50[e]	8	,57	,02		
DOX+E100[f]	8	1,17	,00		

*significant statistical differences were identified when comparing the groups based on DOX-E kidney *Hspa5* mRNA levels (p≤ .000).

Table 7. Results of Tukey HSD test of *Hspa5* qPCR data

Tablo 7. *Hspa5* qPCR verilerinin Tukey HSD testi sonuçları

Tukey's multiple comparisons test	Significant
a vs. b	ns
a vs. c	**
a vs. d	****
a vs. e	ns
a vs. f	***
b vs. c	***
b vs. d	****
b vs. e	ns
b vs. f	****
c vs. d	****
c vs. e	**
c vs. f	ns
d vs. e	****
d vs. f	****
e vs. f	***

a: Control, b: DOX, c: E50, d:E100, e: DOX+E50 f: DOX+E100, ns:p >0.05, *:p < 0.05, **:p < 0.01, ***:p < 0.001 and ****: p < 0.0001

Discussion

DOX, a potent chemotherapeutic agent widely used for treating various cancers, has been associated with the induction of oxidative stress in tissues. The primary mechanism of ROS, leading to a disrupted balance between pro-oxidant and antioxidant factors. DOX-induced oxidative stress results in damage to cellular components such as lipids, DNA, and proteins. The mitochondria, being a major target of DOX, experiences dysfunction, contributing significantly to the overall cardiotoxic effects observed during treatment (Ozturk et al., 2024).

HSPs, specialized proteins synthesized in response to stress, operate as molecular chaperones. Their primary function is to facilitate correct protein folding, thereby safeguarding cellular integrity. (Verma et al. 2021). Furthermore, these proteins play a pivotal role in cellular adaptation by initiating the heat shock response, resulting in heightened expression of stress-responsive genes. This, in turn, enhances the cell's capacity to cope with diverse stressors and uphold cellular homeostasis (Konstantinova et al., 2019). HSPs actively engage in activating signaling pathways that contribute to the regulation of cellular processes in challenging conditions. Moreover, research indicates that HSPs not only boost the immune response but also facilitate tissue regeneration, underscoring their

significance in supporting overall organismal health and cellular resilience (Julier et al. 2017).

Under ordinary circumstances, HSPs are ordinarily expressed at low levels, yet their expression is swiftly triggered when cells face stressors. Their primary function is to act as a protective shield for cells, ensuring the preservation of cellular functionality and integrity (Vasques et al., 2013). The initiation of HSP production is prompted by oxidative stress, arising from the body's antioxidant defenses and an imbalance between ROS production. HSPs actively participate in various cellular processes occurring during and following exposure to oxidative stress (Liu et al., 2022). The noted positive correlation between MDA and HSPs implies that elevated levels of HSP expression may signify an enhanced cellular response to stress (Omidi et al., 2023). Additionally, HSPs demonstrate protective actions in response to oxidative stress. HSPs recognize intracellular redox changes, exert antiapoptotic effects and contribute to the repair of damaged proteins. These mechanisms collectively play a vital role in maintaining cellular homeostasis and shielding against oxidative damage, thereby bolstering survival of cells and the overall resilience during stressful conditions (Kalmar and Greensmith, 2009; Szyller and Bil-Lula, 2021). The production of HSPs serves as a crucial protective mechanism, assisting the body in mitigating the adverse impacts of oxidative stress and ensuring overall health (Kalmar Greensmith, 2009).

In the heat shock response, our experimental results indicated a significant boost in the mRNA expression of noteworthy HSP (*Hspa1a*, *Hsp4a*, and *Hsp5a*) within the DOX group. These findings align with previous study reports that highlight the production of HSPs in response to oxidative stress (Oksala et al., 2014). It is believed that the accumulation of ROS, causing cellular damage, plays a crucial role in activating HSP genes. The exposure of cells to DOX serves as a tissue indicator for the incidence of damage (Lan et al., 2020).

On the other hand, the co-treatment of DOX with esculetin revealed a decrease in the mRNA expression of our researched HSPs. Previous studies have documented the impact of several antioxidants in reducing oxidative damage and HSPs (Yin et al., 2018). The administration of esculetin is suggested that it inhibits HSP expression by stimulating the expression antioxidant enzymes, resulting in the elimination of ROS and enhancing cell viability. It is noteworthy that esculetin treatment resulted in the most significant reduction in HSPs expression. This can be attributed to the high tissue concentrations of esculetin, enhancing antioxidant capacity and effectively eliminating most ROS.

Several studies have been conducted in the literature on the alteration of HSP expression levels with anticancer drugs and DOX. For instance, Sojka et al. (2021) revealed that Manumycin A (MA) induced cell type-specific alterations in the levels of HSPAs. These alterations encompassed a simultaneous increase in the stress-inducible paralogs (HSPA1 and HSPA6) and a decrease in the non-stress-inducible paralog (HSPA2). Interestingly, despite these changes, neither HSPA1 nor HSPA2 were deemed essential for conferring protection against methamphetamine-induced effects in lung cancer cells. This suggests a complex relationship between MA exposure, cell type-specific responses in HSPA levels, and the mechanism underlying cellular protection, emphasizing the need for further exploration of the intricate molecular pathways involved in these processes. Lan et al. (2020) conducted a study to investigate whether Hsp22 could play a effective role during cardiac injury in response to DOX. The study noted a rise in the expression level of Hsp22 in heart tissue treated with DOX. Moreover, the cardiac-specific overexpression of Hsp22 demonstrated a notable improvement in cardiac function, a reduction in cell apoptosis and a dimish in the inflammatory response, in both injured heart tissues and DOX-induced cardiomyocytes, both in vitro and in vivo. Liu et al. (2007) explored the impact of Hsp27 overexpression on heart failure (HF) induced by DOX. Their findings revealed an upregulation of hsp70 and heme oxygenase-1 in the hearts of transgenic subjects following DOX stimulation, as compared to hearts from wild-type individuals. Another study, Liu and colleagues (2019) delved into exploring the potential role of HSP70 in response to HF. Our observations indicated that DOX treatment led to an elevation in circulating HSP70 levels, increased expression of HSP70 in the myocardium, and facilitated its extracellular release within the heart.

Conclusion

In conclusion, an increase in *Hspa1a*, *Hspa4* and *Hspa5* gene levels is observed after DOX treatment compared to the control. This increase is considered as a protection mechanism of cells against stress. Esculetin reduced DOX-induced oxidative stress. The findings of this study offer a theoretical foundation for the potential alleviation of oxidative stress by employing esculetin. In conclusion, an increase in Hspa1a, Hspa4 and Hspa5 gene levels is

observed after DOX treatment compared to the control. This increase is considered as a protection mechanism of cells against stress. Esculetin reduced DOX-induced oxidative stress. The findings of this study offer a theoretical foundation for the potential alleviation of oxidative stress by employing esculetin.

Ethics Committee Approval: Ethics committee approval was received for this study from the ''Local Ethics Committee of Atatürk University Animal Experiments'' (Protocol Number: 2021/4–123).
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Kaynaklar

Albakova, Z., Siam, M. K. S., Sacitharan, P. K., Ziganshin, R. H., Ryazantsev, D. Y., & Sapozhnikov, A. M. (2021). Extracellular heat shock proteins and cancer: New perspectives. *Translational Oncology*, *14*(2). https://doi.org/10.1016/j.tranon.2020.100995

Boopathy, L. R. A., Jacob-Tomas, S., Alecki, C., & Vera, M. (2022). Mechanisms tailoring the expression of heat shock proteins to proteostasis challenges. *Journal of Biological Chemistry*, *298*(5). https://doi.org/10.1016/j.jbc.2022.101796.

Boussada, M., Hammami, I., Ben Ali, R., Ammar, A. B., Alves, M., Oliveira, P. F., Akacha, A. B., Abdelkarim, I. L., Zekri, S., & El May, M. V. (2022). In vivo exposure to a new 2-cyano-2-p-nitrophenyl-N-benzylthioamide decreases doxorubicin-triggered structural damages in the mature testis. *Andrologia*, *54*(11). https://doi.org/10.1111/and.14634.

Ceylan, H., & Erdoğan, O. (2017). Cloning, expression, and characterization of human brain acetylcholinesterase in Escherichia coli using a SUMO fusion tag. *Turkish Journal of Biology*, *41*(1), 77-87. https://doi.org/10.3906/biy-1602-83.

De Freitas, G. B., Penteado, L., Miranda, M. M., Filassi, J. R., Baracat, E. C., & Linhares, I. M. (2022). The circulating 70 kDa heat shock protein (HSPA1A) level is a potential biomarker for breast carcinoma and its progression. *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-17414-6

Desai, V. G., Herman, E. H., Moland, C. L., Branham, W. S., Lewis, S. M., Davis, K. J., George, N. I., Lee, T., Kerr, S., & Fuscoe, J. C. (2013). Development of doxorubicin-induced chronic cardiotoxicity in the B6C3F1 mouse model. *Toxicology and Applied Pharmacology, 266*(1), 109–121. https://doi.org/10.1016/j.taap.2012.10.025

Dos Santos, N. S., Gonçalves, D. R., Balbinot, B., & Visioli, F. (2023). Is GRP78 (Glucose-regulated protein 78) a prognostic biomarker in differents types of cancer? A systematic review and meta-analysis. *Pathology-Research and Practice, 242*. https://doi.org/10.1016/j.prp.2023.154301.

Dubrez, L., Causse, S., Borges Bonan, N., Dumétier, B., & Garrido, C. (2020). Heat-shock proteins: chaperoning DNA repair. *Oncogene, 39*(3), 516-529. https://doi.org/10.1038/s41388-019-1016-y.

Guo, H., Yi, J., Wang, F., Lei, T., & Du, H. (2023). Potential application of heat shock proteins as therapeutic targets in Parkinson's disease. *Neurochemistry International, 162*. https://doi.org/10.1016/j.neuint.2022.105453.

Hassanein, E. H., Sayed, A. M., Hussein, O. E., & Mahmoud, A. M. (2020). Coumarins as modulators of the Keap1/Nrf2/ARE signaling pathway. *Oxidative Medicine and Cellular Longevity,* 2020. https://doi.org/10.1155/2020/1675957

Hu, C., Yang, J., Qi, Z., Wu, H., Wang, B., Zou, F., Mei, H., Liu, J., Wang, W., & Liu, Q. (2022). Heat shock proteins: Biological functions, pathological roles, and therapeutic opportunities. *MedComm, 3*(3), e161. https://doi.org/10.1002/mco2.161

Julier, Z., Park, A. J., Briquez, P. S., & Martino, M. M. (2017). Promoting tissue regeneration by modulating the immune system. *Acta Biomaterialia, 53*, 13–28.

Kalmar, B., & Greensmith, L. (2009). Induction of heat shock proteins for protection against oxidative stress. *Advanced Drug Delivery Reviews, 61*(4), 310–318. https://doi.org/10.1016/j.actbio.2017.01.056.

Kirici, M., Kirici, M., Demir, Y., Beydemir, S., & Atamanalp, M. (2016). The effect of Al+3 and Hg+2 on glucose 6-phosphate dehydrogenase from capoeta umbla kidney. *Applied Ecology and Environmental Research, 14*(2), 253-264. http://dx.doi.org/10.15666/aeer/1402_253264.

Konstantinova, E. V., Chipigina, N. S., Shurdumova, M. H., Kovalenko, E. I., & Sapozhnikov, A. M. (2019). Heat Shock Protein 70 kDa as a Target for Diagnostics and Therapy of Cardiovascular and Cerebrovascular Diseases. *Current Pharmaceutical Design, 25*(6), 710–714. https://doi.org/10.2174/1381612825666190329123924.

Lan, Y., Wang, Y., Huang, K., & Zeng, Q. (2020). Heat Shock Protein 22 Attenuates Doxorubicin-Induced Cardiotoxicity via Regulating Inflammation and Apoptosis. *Frontiers in Pharmacology, 11*, 257. https://doi.org/10.3389/fphar.2020.00257.

Lang, B. J., Guerrero, M. E., Prince, T. L., Okusha, Y., Bonorino, C., & Calderwood, S. K. (2021). The functions and regulation of heat shock proteins; key orchestrators

of proteostasis and the heat shock response. *Archives of Toxicology, 95*(6), 1943-1970. https://doi.org/10.1007/s00204-021-03070-8.

Lee, M. J., Chou, F. P., Tseng, T. H., Hsieh, M. H., Lin, M. C., & Wang, C. J. (2002). Hibiscus protocatechuic acid or esculetin can inhibit oxidative LDL induced by either copper ion or nitric oxide donor. *Journal of Agricultural and Food Chemistry, 50*(7), 2130–2136. https://doi.org/10.1021/jf011296a.

Liu, J. F., Chen, P. C., Ling, T. Y., & Hou, C. H. (2022). Hyperthermia increases HSP production in human PDMCs by stimulating ROS formation, p38 MAPK and Akt signaling, and increasing HSF1 activity. *Stem Cell Research & Therapy, 13*(1), 236. https://doi.org/10.1186/s13287-022-02885-1.

Liu, L., Zhang, X., Qian, B., Min, X., Gao, X., Li, C., Cheng, Y., & Huang, J. (2007). Over-expression of heat shock protein 27 attenuates doxorubicin-induced cardiac dysfunction in mice. *European Journal of Heart Failure, 9*(8),762–769. https://doi.org/10.1016/j.ejheart.2007.03.007

Liu, P., Bao, H. Y., Jin, C. C., Zhou, J. C., Hua, F., Li, K., Lv, X. X., Cui, B., Hu, Z. W., & Zhang, X. W. (2019). Targeting Extracellular Heat Shock Protein 70 Ameliorates Doxorubicin-Induced Heart Failure Through Resolution of Toll-Like Receptor 2-Mediated Myocardial Inflammation. *Journal of the American Heart Association, 8*(20),e012338. https://doi.org/10.1161/JAHA.119.012338

Nguyen, H. C., Frisbee, J. C., & Singh, K. K. (2024). Different Mechanisms in Doxorubicin-Induced Cardiomyopathy: Impact of BRCA1 and BRCA2 Mutations. *Hearts, 5*(1), 54-74.

Oksala, N. K., Ekmekçi, F. G., Ozsoy, E., Kirankaya, S., Kokkola, T., Emecen, G., Lappalainen, J., Kaarniranta, K., & Atalay, M. (2014). Natural thermal adaptation increases heat shock protein levels and decreases oxidative stress. *Redox Biology, 3*, 25–28.

Omidi, A., Nazifi, S., Rasekh, M., & Zare, N. (2023). Heat-shock proteins, oxidative stress, and antioxidants in one-humped camels. *Tropical Animal Health and Production, 56*(1), 29.

Öztürk, N., Karaağaç, M. S., Yeşilkent, E. N., Isıyel, M., Tosun, H., Karataş, H., Ceylan, H., Demir, Y. (2024). Exploring Esculetin's Protective Role:Countering Doxorubicin-Induced Oxidative Stress in Rat Heart. *Journal of Laboratory Animal Science and Practices, 4*(1), 41-49.

Patel, U., Abernathy, J., Savani, B. N., Oluwole, O., Sengsayadeth, S., & Dholaria, B. (2022). CAR T cell therapy in solid tumors: A review of current clinical trials. *EJHaem, 3*, 24-31.

Rawat, P. S., Jaiswal, A., Khurana, A., Bhatti, J. S., & Navik, U. (2021). Doxorubicin-induced cardiotoxicity: An update on the molecular mechanism and novel therapeutic strategies for effective management. *Biomedicine and Pharmacotherapy, 139*, 111708.

Rehati, A., Abuduaini, B., Liang, Z., Chen, D., & He, F. (2023). Identification of heat shock protein family A member 5 (HSPA5) targets involved in nonalcoholic fatty liver disease. *Genes and Immunity, 24*(3), 124–129.

Shan, Q., Ma, F., Wei, J., Li, H., Ma, H., & Sun, P. (2020). Physiological functions of heat shock proteins. *Current Protein and Peptide Science, 21*(8), 751-760.

Shang, B. B., Chen, J., Wang, Z. G., & Liu, H. (2021). Significant correlation between HSPA4 and prognosis and immune regulation in hepatocellular carcinoma. *PeerJ, 9*, e12315.

Sojka, D. R., Hasterok, S., Vydra, N., Toma-Jonik, A., Wieczorek, A., Gogler-Pigłowska, A., & Scieglinska, D. (2021). Inhibition of the Heat Shock Protein A (HSPA) Family Potentiates the Anticancer Effects of Manumycin A. *Cells, 10*(6), 1418.

Szyller, J., & Bil-Lula, I. (2021). Heat Shock Proteins in Oxidative Stress and Ischemia/Reperfusion Injury and Benefits from Physical Exercises: A Review to the Current Knowledge. *Oxidative Medicine and Cellular Longevity, 2021*, 6678457.

Tian, Z., Yang, Y., Yang, Y., Zhang, F., Li, P., Wang, J., Yang, J., Zhang, P., Yao, W., & Wang, X. (2020). High cumulative doxorubicin dose for advanced soft tissue sarcoma. *BMC cancer, 20*(1), 1139.

Tien, Y. C., Liao, J. C., Chiu, C. S., Huang, T. H., Huang, C. Y., Chang, W. T., & Peng, W. H. (2011). Esculetin ameliorates carbon tetrachloride-mediated hepatic apoptosis in rats. *International Journal of Molecular Sciences, 12*(6), 4053–4067.

Toraman, A., Toraman, E., Özkaraca, M., & Budak, H. (2022). Increased nociceptive sensitivity is associated with periodontal inflammation and expression of chronic pain genes in gingival tissues of male rats. *Chemico-Biological Interactions, 366*, 110128.

Tukaj, S. (2020). Heat shock protein 70 as a double agent acting inside and outside the cell: insights into autoimmunity. *International Journal of Molecular Sciences, 21*(15), 5298.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3—new capabilities and interfaces. *Nucleic acids research, 40*(15), e115-e115.

Van der Zanden, S. Y., Qiao, X., & Neefjes, J. (2021). New insights into the activities and toxicities of the old

anticancer drug doxorubicin. *The FEBS journal, 288*(21), 6095-6111.

Vasques, M. T., Alves, M. A., Benetti, C., Aranha, A. C., Zezell, D. M., & Corrêa, L. (2013). Temperature measurement and Hsp47 immunoexpression in oral ulcers irradiated with defocused high-energy diode laser. Journal of Photochemistry and Photobiology. B, *Biology, 118*, 42–48.

Yesilkent, E. N., & Ceylan, H. (2022). Investigation of the multi-targeted protection potential of tannic acid against doxorubicin-induced kidney damage in rats. Chemico-*Biological Interactions, 365*, 110111.

Yin, B., Tang, S., Sun, J., Zhang, X., Xu, J., Di, L., Li, Z., Hu, Y., & Bao, E. (2018). Vitamin C and sodium bicarbonate enhance the antioxidant ability of H9C2 cells and induce HSPs to relieve heat stress. *Cell Stress and Chaperones, 23*(4), 735–748.