MCBU SBED
MANİSA CELAL BAYAR ÜNİVERSİTESİ SAĞLIK BİLİMLERİ ENSTİTÜSÜ DERGİSİ
MANISA CELAL BAYAR UNIVERSITY JOURNAL OF INSTITUTE OF HEALTH SCIENCE
ISSN: 2147-9607

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RESEARCH ARTICLE
CBU-SBED, 2025, 12 (4): 499-505

Protective Effects of Gallic Acid Against Chronic Restraint Stress-Induced Histological Alterations of Skin

Kronik Kısıtlama Stresinin Deride Neden Olduğu Histolojik Değişikliklere Karşı Gallik Asidin Koruyucu Etkileri

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Gönderim Tarihi / Received: 20.01.2025
Kabul Tarihi / Accepted: 27.05.2025
DOI: 10.34087/cbusbed.1623924

Öz

Giriş ve Amaç: Stres, insanların yaşadığı en önemli sorunlardan biridir. Günümüzün pandemisi olarak nitelendirilen stres, pek çok hastalığın patogenezinde rol oynar. Hipotalamus-Hipofiz-Adrenal (HPA) ekseninin stres karşısında kronik aktivasyonu, dokulardaki inflamatuar yanıtın ve reaktif oksijen türlerinin (ROS) artmasına neden olur. Atopik dermatit, sedef hastalığı gibi birçok inflamatuar deri hastalığı stres ile tetiklenir ya da kötüleşir. Çalışmamızda fiziksel ve mental stres modeli olan kronik kısıtlama stresi kullanılmış ve bu modelin sıçan derisinde neden olduğu hasara karşı gallik asit (GA)'in koruyucu etkisi morfolojik düzeyde araştırılmıştır.

Gereç ve Yöntemler: Denekler 4 eşit gruba ayrılmıştır (n=7): Kontrol (K), Gallik Asit (GA), Kronik Stres (KS) ve Kronik Stres+Gallik Asit (KS+GA). Her gruptan alınan histolojik kesitler hematoksilen-eozin (H-E), Masson trikrom ve toluidin mavisi ile boyanmıştır.

Bulgular: KS grubunda epidermal kalınlıkta azalma, epidermal hücre morfolojilerinde dejenerasyonlar, kollajen bütünlüğünde kayıplar, mast hücre sayısı ve anti-IL-6 immünohistokimyasal pozitivite artışı görüldü. KS+GA grubunda ise epidermal kalınlık, inflamasyon ve kollajen organizasyonunun K grubu ile benzerlik gösterdiği görüldü. Anti-IL-6 immünohistokimyasal pozitivitenin KS grubuna kıyasla anlamlı düzeyde azaldığı görüldü.

Sonuç: Bulgularımıza göre gallik asitin, kronik hareketsizlik stresinin deride meydana getirdiği hasarı morfolojik olarak azalttığı görülmüştür. Elde edilen bulgular, ileride yapılacak çalışmalarla birlikte gallik asidin stres kaynaklı deri hasarlarında koruyucu bir ajan olarak değerlendirilebileceğini göstermektedir.

Anahtar kelimeler: Deri, Kronik Kısıtlama stresi, Gallik asit

Abstract

Aim: Stress is a significant problem that people experience. It has been called the pandemic of our time and plays a role in the pathogenesis of many diseases.

Chronic activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to stress leads to increased tissue inflammation and reactive oxygen species (ROS). Many inflammatory skin conditions, such as atopic dermatitis and psoriasis, are triggered or exacerbated by stress. In our study, chronic restraint stress, a physical and mental stress model, was used to investigate the protective effect of gallic acid (GA) against the damage induced by this model in rat skin at the morphological level.

Method: Subjects were divided into four equal groups (n=7): Control (C), Gallic Acid (GA), Chronic Stress (CS**),** and Chronic Stress+Gallic Acid (CS+GA). Histologic sections from each group were stained with hematoxylin-eosin (H-E), Masson trichrome, and toluidine blue.
Results: In the CS group, there was a decrease in epidermal thickness, degeneration in epidermal cell morphology, and a detachment of collagen integrity. Concurrently, there was an increase in mast cell number and anti-IL-6 immunohistochemical positivity. In the CS+GA group, epidermal thickness, inflammation, and collagen structure parameters were similar to those of the CS group. Anti-IL-6 immunohistochemical positivity was significantly decreased compared to the CS group.
Conclusion: According to our findings, gallic acid morphologically reduced the damage caused by chronic immobility stress on the skin. Given these findings, different studies may use gallic acid as a protective adjuvant in stress-induced skin damage.

Keywords: Skin, Chronic Restraint Stress, Gallic acid

1. Introduction

Stress is a physiological and psychological state frequently encountered by living beings at every stage of life. Considered the "disease of the modern age," stress can be a source of motivation in short durations [1]. However, when stress is recurrent or prolonged, it becomes chronic and contributes to the pathogenesis of various diseases [2,3]. Chronic stress disrupts homeostasis by increasing circulating glucocorticoid concentrations [4,5]. Although the activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis in the face of stress is an adaptation to maintain the homeostasis of the organism; Glucocorticoid, which reaches high levels in the circulation, causes an increase in the inflammatory response and ROS in the tissues [6].

The body's largest organ, the skin, functions as a homeostatic barrier between the internal and external environments and is constantly exposed to external stressors [7]. Studies have shown that increased systemic glucocorticoids due to stress negatively affect epidermal cell proliferation and homeostasis [4,8–10]. Clinically, stress is known to trigger inflammatory skin conditions such as psoriasis and atopic dermatitis [11–16].

Corticotropin-releasing hormone (CRH), a potent vasodilator involved in the HPA axis and stress response, is expressed in the epidermal cells of both humans and rodents [7]. CRH stimulates mast cell degranulation, which triggers the release of chemokines and cytokines involved in inflammation [17–20]. Chemokines and cytokines, such as IL-1α and IL-6 produced by keratinocytes and mast cells, exacerbate inflammation by promoting the migration of inflammatory cells, differentiation of keratinocytes, and production of additional cytokines [19,21–24].

The restraint stress model used in our study is a well-established model for simulating physical stress in experimental settings and provides valuable insights into stress-mediated pathophysiological mechanisms [21,25–29].

Gallic acid (GA) is a phenolic compound in natural products known for its antioxidant and anti-inflammatory effects [30]. The therapeutic effects of GA have been documented in various studies involving inflammatory diseases and skin conditions [31,32]. GA attenuates the inflammatory response by reducing the release of inflammatory cytokines, chemokines, and adhesion molecules [33]. The anti-inflammatory mechanisms of GA are reported to involve the inhibition of mast cell degranulation and interleukin-6 (IL-6) signaling pathways [34–36].

In this study, we investigated the therapeutic effects of gallic acid on skin homeostasis under chronic restraint stress at a morphological level.

2. Material Method

2.1 Animals

The subjects were 8-week-old adult male rats housed at 22-23^{0}C and 60% humidity in a 12:12 h light/dark cycle. Standard pellet feed and ad libitum nutrition were provided. The Hamidiye Experimental Animal Production and Research Laboratory of the University of Health Sciences (2024-04/10) approved all experimental protocols.

2.2 Experimental Groups

A total of 28 male Sprague-Dawley rats were allocated into four distinct groups, each consisting of seven subjects:

Control (C): No intervention was administered (n=7)

Gallic Acid (GA): Rats received a daily oral gavage of 20 mg/kg gallic acid for 15 days (n=7)

Chronic Stress (CS): Rats were exposed to 6 hours of daily immobilization stress for 15 days (n=7)

Chronic Stress + Gallic Acid (CS + GA): Rats received 20 mg/kg gallic acid via oral gavage before each 6-hour immobilization stress session over 15 days (n=7).

2.3 Experimental Protocol

The experimental protocol involved utilizing 15x5x5-sized plexiglass stress cages to implement the immobilization stress model. Rats were secured by their tails within these cages and subjected to immobilization stress daily (09:00-15:00) for 15 days. Freshly prepared gallic acid (Sigma, Germany) dissolved in distilled water was orally administered via oral gavage to subjects in the GA and CS+GA groups at a dosage of 20 mg/kg per rat daily.

2.4 Tissue Collection

After completing the experimental protocol, the subjects were euthanized while under ketamine-xylazine anesthesia (Ketalar Xylazinbio). Skin samples were

taken from the dorsal region (2 cm^2) fixed in neutral buffered formalin (NBF) and prepared for histological and immunohistochemical analyses.

2.5 Histological Preparation

Skin samples, including the hypodermis, were fixed in NBF and then routinely processed for paraffin embedding. Sections of approximately five µm thickness were obtained using a Microm HM325 microtome from Thermo Scientific, Germany. For morphologic evaluation, sections were stained with H-E, and Masson trichrome stains. Toluidine blue was used to visualize mast cell morphology in the tissue. The sections were evaluated for the presence of mature granulated mast cells and degranulated mast cells. In sections taken at 10-section intervals, five different fields of each section at an x400 microscopic magnification were counted by a two-blinded observer. Mast cell was quantified as the number of cells per unit area [37]. Inflammatory cell (leukocyte) infiltration in the dermis was evaluated based on the accumulation of mononuclear cells, categorized as mild, moderate, or severe. For this parameter, a semi-quantitative scoring system ranging from 0 (normal) to 3 (severe infiltration) was used in fifteen random fields in comparison with control groups [37,38]. Epidermal thickness was defined as the distance (in µm) from the granular layer's top to the basal layer's bottom. Epidermal thickness was measured in fifteen microscopic field, and the average value was calculated for each group [37]. The specimens were examined and photographed, blinded by the same histologists, using a Zeiss Axio Scope A1 light microscope with a digital camera attachment.

2.6 Immunohistochemical Preparation

Tissues were fixed in NBF for 48 hours. Routine light microscopy was performed, and sections with a mean thickness of 3-5 µm were taken from the paraffin-blocked skin tissues on a positively charged slide. The sections were deparaffinized in xylene and dehydrated in a decreasing alcohol series. Then, each slide was placed in citrate buffer (pH: 6, DIAPATH Lot: 2019x05445) at 90-95 ^{0}C in a microwave oven for antigen retrieval. After treatment, they were incubated with a protein-blocking solution with 3% hydrogen peroxide (H_2O_2). Samples were incubated overnight at +4°C with the IL-6 primary antibody (Abcam, AB9324, dilution 1/200) without prior washing. After overnight incubation, the sections were incubated with a mouse specifying complement reagent to enhance antigen-antibody complex stabilization, followed by a 15-minute incubation with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam, AB64261, Lot: 10444484-1). After diaminobenzidine (DAB) and hematoxylin-light green counterstaining, sections were passed through an ascending alcohol series and covered with the mounting medium (Merck, Germany, Lot: HX90503161). All dilutions and thorough washes between steps were performed using PBS, and all steps were performed at room temperature unless otherwise specified. The images obtained were examined and photographed under a light microscope (Zeiss Axiocam ScopeA1, UK). The expression of IL-6 in immunohistochemically labelled skin sections was evaluated, and staining intensity was scored on a scale from 0 to 3 (0: no expression, 1: weak, 2: moderate, 3: strong) [39].

3. Results and Discussion

3.1 Results

3.1.1 Light Microscopy

In the Control (C) and Gallic Acid (GA) groups, epidermal cells exhibited normal morphology, and collagen fibers in the dermis were arranged in an irregular, dense connective tissue pattern (Figure 1 A, B, E, F). In the Chronic Stress (CS) group, thinning and separation of collagen fibers were observed, along with decreased epidermal thickness (Figure 1 I, J). In the Chronic Stress + Gallic Acid (CS+GA) group, Similar to groups C and GA, collagen fibers were observed in normal distribution and order.

Epidermal thickness measurements revealed a significantly decrease in the CS group compared to the C and GA groups (*p<0.05). However, the CS+GA group showed a significantly increase in epidermal thickness compared to the CS group (*p<0.05), (Table 1), (Figure 1 M).

Figure 1. ***Epithelial thickness and morphological changes in basal epithelial cells (thin arrow), inflammatory cell infiltration (**) (A, E, I, M, inset A, E, I, M); H-E, x200 microscope magnification, x400 microscope magnification inset images; connective tissue, collagen fibers (star), (B, F, J, N), Masson's trichrome, x200 microscope magnification; dermal mast cells (arrowhead), (C, G, K, O), toluidine blue; x400 microscope magnification; IL-6 immunopositive areas (thick arrow), (D, H, L, P), anti-IL-6 IHC, × 200 microscope magnification in the experimental groups.***

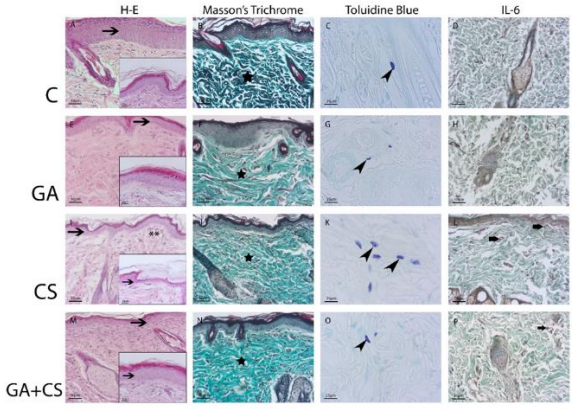
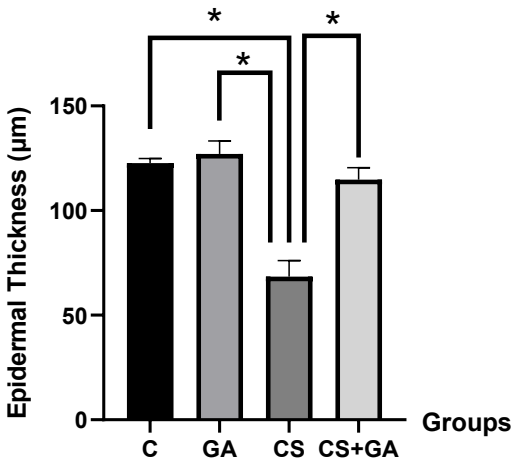
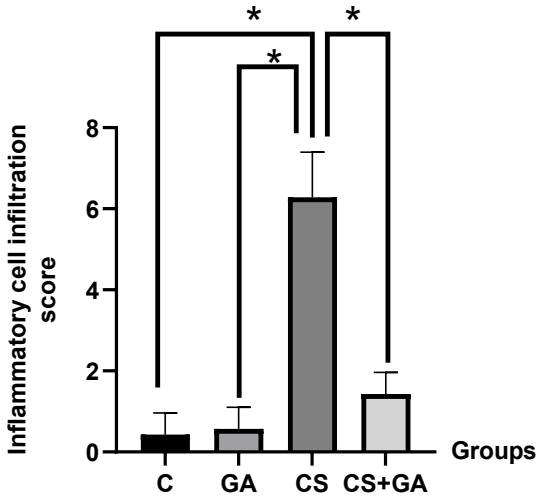


Table 1. *Comparison of epidermal thickness measurements between groups.* (*p<0.05)



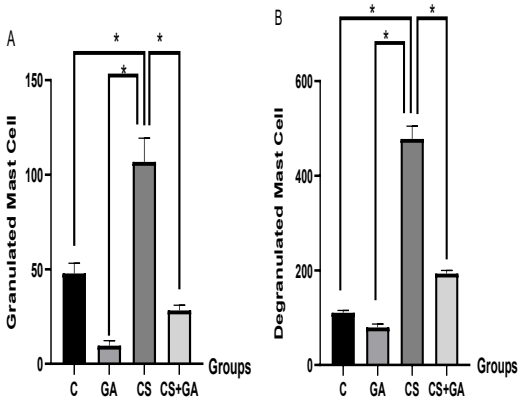
Inflammatory cell (leukocyte) infiltration that was slight, moderate, or severe was characterized by diffused infiltration of mononuclear inflammatory cells into the dermis compared with the controls. It was detected that there was no inflammatory cell infiltration in the dermis in groups C and GA (Table 2), (Figure 1 A, E). A significant increase in inflammatory cell infiltration was exhibited in the CS group in comparison with the C and GA groups. (*p<0.05), (Table 2), (Figure 1 I). In the CS+GA group, inflammatory cell infiltration was observed to decrease significantly compared to the CS group (*p<0.05), (Table 2), (Figure 1 M).

Table 2. *Comparison of inflammatory cell infiltration between groups.* (*p<0.05)



In groups C and GA, a few scattered intact mast cells were seen (Figure 1 C, G). It was revealed that the number of granulated and degranulated mast cells increased significantly in the CS group. It was observed that active degranulated mast cells increased in number compared to granulated mast cells (*p<0.05), (Table 3 A, B), (Figure 1 K). It was detected that the number of mast cells in the CS+GA group decreased at a significant level, similar to the C and GA groups, and intact cells were more than active cells (*p<0.05), (Table 3 A, B), (Figure 1 O). The groups quantitative granulated and degranulated mast cell analyses are shown in Tables 3A and 3B.

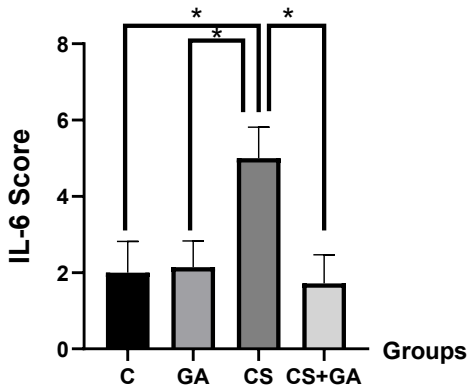
Table 3A ve 3B. ***Comparison of granulated and degranulated mast cell numbers between groups.*** **(*p<0.05)**



3.1.2 Immunohistochemical Staining of IL-6

In groups C and GA, anti-IL-6 IHC revealed mild immunopositive markings primarily in dermal inflammatory cell infiltrates and vascular endothelial areas (*p<0.05), (Table 4), (Figure 1 D, H). In the CS group, immunopositive markings were more intense and widespread, showing a significant increase (*p<0.05), (Table 4), (Figure 1 L). In the CS+GA group, similar to the C and GA groups, the density and prevalence of immunopositive inflammatory and vascular regions were detected to decrease at a significant level (*p<0.05), (Table 4), (Figure 1 P).

Table 4. *Comparison of anti-IL-6 densities between groups.* (*p<0.05)



3.1.3 Statistical Analysis

The normality of data distribution was assessed using the Shapiro–Wilk test, while the homogeneity of variances was evaluated with Levene's test. Depending on these assumptions, group comparisons were performed using Brown–Forsythe and one-way analysis of variance (ANOVA). For post hoc analyses, the Dunnett T3 or Tukey's test was applied, depending on the equality of variances. The data are presented as the mean ± standard deviation (SD). A p-value of less than 0.05 was

considered to be statistically significant. All statistical analyses were conducted using SPSS software (Version 26.0; IBM Corp., Armonk, NY, USA).

3.2 Discussion

The skin is a large, multifunctional organ that covers the body's surface. In addition to its role as a protective barrier against external factors, it also serves as a sensory interface that is essential for maintaining homeostasis. Stress disrupts homeostasis, impacting various organs. Among these, the skin is particularly vulnerable to the adverse effects of stress [40]. Stress-related skin diseases include atopic dermatitis, prurigo nodularis, and psoriasis. Clinically, these conditions are also called inflammatory skin diseases [40].

ROS plays a key role in the development and exacerbation of many inflammatory diseases [41]. Under chronic stress, elevated ROS levels accumulate in tissues, and the severity of inflammatory diseases increases in prolonged stress conditions [42]. Mast cells play a key role in inflammation. Located in the dermis near the blood vessels, these cells facilitate the migration of immüne cells into connective tissue. Therefore, mast cell activation is an essential marker for detecting tissue inflammation. Growth factors released under the influence of stress increase the number of mast cells and trigger their degranulation [40–45]. Active during increased stress, CRH stimulates mast cell degranulation [17–20]. In a study using a restraint stress model, an increase in the number of degranulated mast cells was noted in the stress group [46]. Similarly, in our study, the inflammatory score and number of degranulated mast cells were significantly higher in the CS group than in the C and GA groups.

When mast cells degranulate, they release pro-inflammatory cytokines into the extracellular environment, including IL-6. The secretion of pro-inflammatory cytokines like IL-6 exacerbates the inflammatory microenvironment. Several studies have investigated the modulatory effects of gallic acid on IL-6 expression, particularly in the context of inflammation-related pathologies. [47,48]. Similarly, in our study, IL-6 immunopositive cells were significantly higher in the CS group than in the C and GA groups. This finding supports that IL-6 released from mast cells in the CS group increases and subsequently triggers inflammation.

With the gallic acid supplementation, the higher number of granulated mast cells compared to degranulated mast cells in the treatment groups indicates that gallic acid may reduce mast cell activation. Similarly, toluidine blue staining used to evaluate mast cell morphology and number indicated that mast cells may play a triggering role in the observed inflammatory response. The ratios observed in our granulated and degranulated mast cell analysis suggest that gallic acid may influence these pathways.

Excessive ROS levels in the body enhance inflammation and activate cell death mechanisms. The primary barrier function in the skin is provided by the structure formed by lipids in the stratum corneum layer of the epidermis and keratinocytes. Increased ROS, particularly in keratinocytes, triggers oxidative damage, leading to adverse effects such as lipid peroxidation, protein denaturation, and DNA damage. Epidermal thinning, disruption of the stratum corneum's integrity, and alterations in the organization of intercellular lipids may also be observed. Under these circumstances, the skin barrier is damaged, increasing transepidermal water loss (TEWL), resulting in skin dryness, inflammation, and increased barrier permeability to external pathogens [49]. Inflammatory cytokines are secreted in the disrupted skin barrier, advancing inflammation. Some of these cytokines stimulate the activation of mast cells. The increased number of mast cells, which secrete proinflammatory cytokines, induces the migration of inflammatory cells into the dermis. However, due to chronic stress, the overactivation of mast cells increases inflammation, leading to a systemic vicious cycle and the accumulation of ROS in the tissue. Elevated ROS and glucocorticoid levels under chronic stress have been shown to decrease epidermal proliferation, thereby damaging the skin barrier [50,51]. Studies in the literature that modeled atopic dermatitis with restraint stress reported hyperkeratosis in the epidermis, while some studies also found that chronic stress disrupts proliferation and reduces epidermal thickness [52].

In our study, no dermatitis was observed in the stressed group; however, the epidermal thickness was similar to the studies in the literature, and the epidermal thickness of the CS group was significantly reduced compared to the C and GA groups. We believe the increased ROS under chronic stress damages the membranous components of mitotically active basal cells, disrupting epithelial turnover. Our findings of morphological damage in basal cells also support this claim. The reduced epidermal thickness may be due to damaged epithelial cells and disrupted epithelial turnover.

Under normal homeostasis conditions, the body combats oxidative damage caused by increased ROS through a strong endogenous antioxidant system. The skin, constantly exposed to internal and external stressors, requires high antioxidant defense. Under chronic stress, the skin needs exogenous antioxidant replacement due to insufficient antioxidant capacity to counteract the increased oxidative damage [50,51]. GA, a phenolic acid in many natural products, neutralizes ROS and enhances the body's endogenous antioxidant defense. Besides its antioxidant effect, GA also has anti-inflammatory properties [30].

Zamudio-Cuevas et al. (2021) evaluated the effect of GA on proinflammatory cytokines in vitro and found that GA reduced IL-6 secretion. Hu and Zhou investigated the impact of GA administered orally by gavage in a study in which they created an atopic dermatitis model in mice. They reported that GA reduced inflammatory cytokines and increased epidermal thickness, showing a therapeutic effect on the disease [32].

In our study, the treatment group showed a decrease in mast cell numbers and IL-6 immunopositivity compared to the CS group. The inflammation score of the same

group was also lower than that of the stress group. The reduced epidermal thickness in the CS group showed an inverse correlation with inflammation and increased in the CS+GA group. Additionally, histopathological examination revealed that stress-induced cell damage in the epidermis improved in the GA-treated group. These findings suggest that the increase in epidermal thickness is associated with an increase in healthy cell proliferation. In layers where collagen fiber distribution and epidermal cell morphology were disrupted under stress, collagen fibers, and epidermal cells were observed to return to a healthy and normal arrangement after GA treatment.

According to our findings, GA used against skin damage caused by restraint stress reduced mast cell activation and inflammation in the skin. This reduced the destructive effects of increased ROS due to inflammation in tissues. Thus, epithelial cells were preserved, and epidermal thickness increased, approaching that of healthy tissue.

4. Conclusion

Our study determined that the applied stress model caused histological damage to the skin. GA application preserved the typical skin morphology by reducing oxidative stress and inflammation caused by stress. The findings obtained in our study regarding the use of GA to mitigate the damage caused by chronic stress modeled in the skin serve as a foundational research basis for studies that can be supported with different analytical methods.

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