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Immunohistochemical analysis of HSP-70 and IL-6 expression in rooster testes treated with *Cucurbita moschata* seed extract under heat stress condition

S. E. Rochmi[1,]* M. S. Sofyan[1], A. B. Achmad[1], H. Pertiwi[1], G. Residiwati[2], I. S. Ihsan[3,4], H. S. A. Tuska[5], and Supriyadi[6]

[1]Veterinary Technology Program, Department of Health, Faculty of Vocational Studies, Universitas Airlangga, Surabaya 60115, Indonesia
[2]Laboratory of Embryology, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang 65145, Indonesia
[3]Department of Basic Veterinary Medicine, Faculty of Health, Medicine and Natural Sciences, Universitas Airlangga, Banyuwangi, 68425, Indonesia
[4]Stem Cell Research and Development Center, Universitas Airlangga, Surabaya 60115, Indonesia
[5]Department of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang 65145, Indonesia
[6]Magister Program of Biology Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya 60115, East Java, Indonesia
**Corresponding e-mail: eliana.rochmi@vokasi.unair.ac.id*

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ABSTRACT

Heat stress is a major environmental factor that disrupts reproductive function in poultry, primarily by triggering oxidative stress and inflammation in testicular tissue. This study evaluated the influence of *Cucurbita moschata* (pumpkin seed) extract in mitigating the adverse effect of heat stress in roosters testes by regulating key markers, heat shock protein 70 (HSP70) and interleukin-6 (IL-6). Twenty-five adult roosters, aged one year, were divided into five groups: a non-stressed control group, a heat-stressed group without treatment, and three heat-stressed groups treated orally with 1600, 3200, or 6400 mg/kg body weight of the extract for 21 consecutive days. Immunohistochemical analysis revealed that heat stress led to marked increases in HSP-70 and IL-6 expression in the testes, reflecting cellular stress and inflammatory responses. Notably, administration of *Cucurbita moschata* extract attenuated these responses in a dose-dependent manner, with the 3200 and 6400 mg/kg doses showing the most significant reduction in protein expression. These findings highlight the potential role of *Cucurbita moschata* as a natural therapeutic agent to protect male reproductive function under heat stress conditions through its antioxidant and anti-inflammatory properties.

Keywords: Antioxidant, Cucurbita moschata, IL-6, HSP-70, Zero Hunger

INTRODUCTION

Heat stress remains one of the most critical environmental challenges affecting poultry health and productivity, especially in tropical and subtropical climates. Prolonged exposure to high ambient temperatures impairs the birds' thermoregulatory capacity, triggering a cascade of physiological disturbances including oxidative stress, systemic inflammation, and reproductive dysfunction (Lara and Rostagno, 2013). The testes in male poultry are highly susceptible to fluctuations in temperature, particularly during episodes of heat stress. These thermal conditions have been shown to disrupt hormonal pathways, interfere with spermatogenesis, and modulate the levels of proteins involved in stress and inflammation pathways in testicular tissue (Maroto *et al*., 2025).

One of the primary cellular responses to thermal stress involves the heightened expression of heat shock protein 70 (HSP-70), a molecular chaperone that plays an essential protective role. This protein is rapidly synthesized under stress conditions to help maintain protein homeostasis, facilitate the refolding of denatured proteins, and in some cases, participate in the activation of cell death pathways (Balakrishnan *et al*., 2023; Liu *et al.*, 2024). Interleukin-6 (IL-6) is a crucial pro-inflammatory cytokine whose levels generally increase in response to oxidative stress or tissue injury, making it a reliable marker of inflammation during heat stress conditions (Tanaka *et al*., 2014). In humans, increased levels of IL-6 and heat shock protein 70 (HSP-70) in testicular tissue have been associated with impaired reproductive function due to prolonged exposure to high environmental temperatures (Al-Zghoul and Saleh, 2020).

In recent times, there has been growing interest in the use of natural antioxidants to help offset the adverse effects of heat stress in poultry. One particulary promising source is pumpkin seeds (*Cucurbita moschata*), which are rich in bioactive composites similar as tocopherols, flavonoids, polyunsaturated fatty acids, and essential minerals like zinc. These factors are known for their strong antioxidant and anti-inflammatory properties (Rochmi and Pertiwi, 2020). While several studies in mammals have shown the benefits of these composites in reducing oxidative stress and supporting reproductive health, similar studies in poultry, particularly under heat stress, remain limited.

In light of this context, the present study clarified the potential of *Cucurbita moschata* (pumpkin seed) extract to alleviate heat stress-induced damage in rooster testes. The analysis focused on the extract's ability to modulate specific markers: the stress response protein Heat Shock Protein 70 (HSP70) and the proinflammatory cytokine Interleukin-6 (IL-6) The findings are anticipated to contribute to the development of phytogenic feed complements that can enhance reproductive adaptability in flesh facing climate-related thermal challenges.

MATERIALS AND METHODS

Ethical Approval

Ethical approval for all animal procedures was obtained from the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia (Approval No: 1.KEH.162.10.2023). The study was conducted in accordance with Indonesian national regulations and international standards for the ethical treatment of animals in scientific research.

Pumpkin Seed Extraction

Pumpkin seeds (*Cucurbita moschata*) were sourced from Tuban Regency, East Java, Indonesia. The seeds were air-dried and ground into fine powder using a commercial electric grinder (Miyako BL-152 GF, PT. Kencana Gemilang, Jakarta, Indonesia). Extraction was conducted by maceration as previously described, with slight modifications. Specifically, 100 g of powdered seeds were immersed in 500 mL of 96% ethanol (Brataco Chemika, Surabaya, Indonesia) for 72 hours at room temperature with intermittent stirring. The mixture was filtered through Whatman No. 1 filter paper (Cytiva, UK), and the filtrate was concentrated under reduced pressure at 40°C using a rotary evaporator (Heidolph Laborota 4000, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany), yielding a semi-solid crude extract.

Experimental Design and Animal Treatment

Twenty-five healthy adult male domestic chickens (*Gallus domesticus*), aged approximately one year and weighing 1.5–2.0 kg, were used. Birds were housed individually in wire cages under standardized husbandry and environmental conditions and provided feed and water ad libitum.

Chickens were randomly divided into five experimental groups (n = 5 per group): Negative Control (C-): No heat stress, no extract. Positive Control (C+): Heat stress only (37–38°C, 75–80% RH, 8 hours/day for 21 days). Treatment 1 (T1): Heat stress + *C. moschata* extract 1600 mg/kg BW. Treatment 2 (T2): Heat stress + *C. moschata* extract 3200 mg/kg BW. Treatment 3 (T3): Heat stress + *C. moschata* extract 6400 mg/kg BW.

The extract was administered orally once daily via gavage for 21 consecutive days. Heat stress was applied using a controlled-environment chamber. At the end of the treatment period, all animals were humanely euthanized by cervical dislocation following AVMA guidelines. Testes were promptly excised, rinsed with phosphate-buffered saline (PBS), and fixed in 10% neutral buffered formalin for histopathological and immunohistochemical analysis.

Study Period and Location

The study was conducted from April to June 2024. Extraction procedures were carried out in the Laboratory of the Faculty of Pharmacy, Universitas Airlangga, while immunohistochemical analyses were performed at the Veterinary Technology Laboratory, Faculty of Vocational Studies, Universitas Airlangga, Surabaya, Indonesia.

Immunohistochemistry and Protein Expression Scoring

Formalin-fixed tissues were processed and embedded in paraffin. Sections (5 µm thick) were mounted on poly-L-lysine-coated slides. After deparaffinization and rehydration, antigen retrieval was performed using citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Primary antibodies used included: Rabbit polyclonal anti-HSP-70 (sc-33575, Santa Cruz Biotechnology, Dallas, TX, USA). Rabbit polyclonal anti-IL-6 (sc-1265, Santa Cruz Biotechnology, Dallas, TX, USA).

Antibodies were diluted according to manufacturer instructions and incubated overnight at 4°C in a humidified chamber. Detection was performed using HRP-conjugated secondary antibody (goat anti-rabbit IgG-HRP, sc-2004, Santa Cruz Biotechnology, Dallas, TX, USA), followed by visualization with DAB (3,3′-diaminobenzidine) chromogen kit (Biocare Medical, Pacheco, CA, USA). Slides were counterstained with hematoxylin, dehydrated, and mounted for light microscopy.

Protein expression levels of HSP-70 and IL-6 were assessed semi-quantitatively using a modified Remmele Immunoreactive Score (IRS), based on staining intensity and the percentage of positive cells. Two independent, blinded observers evaluated the sections under 400× magnification.

Statistical Analysis

Statistical analysis was conducted using SPSS software version 26.0 (IBM Corp., Armonk, NY, USA). IRS scores were expressed as mean ± standard deviation (SD). Comparisons among groups were made using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

HSP-70 Expression in Rooster Testes

Immunohistochemical (IHC) analysis was conducted to assess HSP-70 expression in rooster testicular tissues, using the modified Remmele Immunoreactive Score (IRS). The Kruskal–Wallis test revealed a statistically significant difference in HSP-70 expression among the experimental groups ($p < 0.05$), and further pairwise comparisons were performed using the Mann–Whitney U test.

As presented in Table 1, the highest HSP-70 expression was observed in the positive control group (C+), which was subjected to heat stress without treatment (7.52 ± 1.044). Treatment with *Cucurbita moschata* seed extract resulted in a dose-dependent decrease in HSP-70 expression. The group treated with 1600 mg/kg BW (T1) showed a moderate reduction (5.56 ± 0.841),

Table 1. Mean IRS Scores of HSP-70 Expression for Each Treatment

Treatment	HSP-70 Expression Score (Mean ± SD)
C− (Negative Control)	1.28 ± 0.521
C+ (Positive Control)	7.52 ± 1.044
T1 (Pumpkin seed extract 1600 mg/kg BW)	5.56 ± 0.841
T2 (Pumpkin seed extract 3200 mg/kg BW)	3.20 ± 1.754
T3 (Pumpkin seed extract 6400 mg/kg BW)	2.84 ± 0.698

Table 2. Mann-Whitney Test Between Treatment Groups on HSP-70 Expression

Group	Compared to	Sig (p-value)
C+	T1	0.028*
	T2	0.009*
	T3	0.009*
	C−	0.009*
T1	T2	0.047*
	T3	0.009*
	C−	0.009*
T2	T3	0.672
	C−	0.027*
T3	C−	0.008*

*Significant at $p < 0.05$

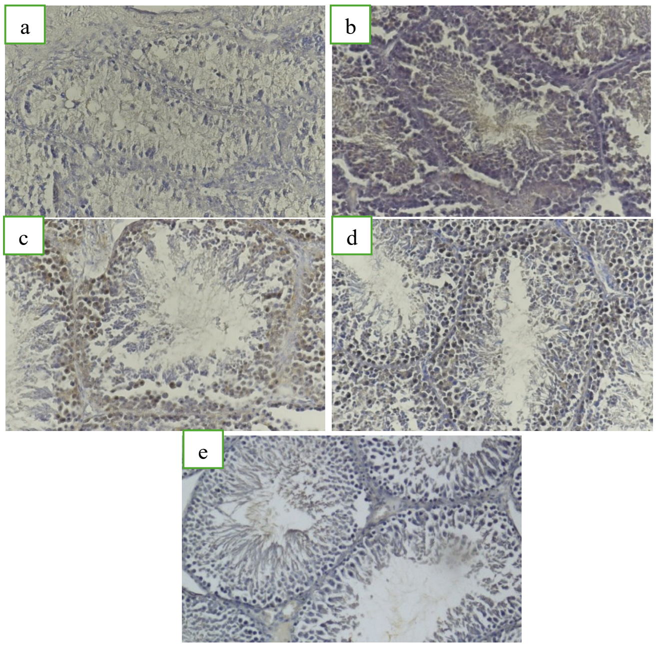

Figure 1. Expression of HSP-70 in chicken testes a. C+ (positive control), b. T1 (pumpkin seed extract 1600 mg/BW), c.T2 (pumpkin seed extract 3200 mg/BW), d. (pumpkin seed extract 6400 mg/BW), e. Negative control. (Nikon Eclipse Ci; 400×).

followed by further decreases in the 3200 mg/kg BW group (T2; 3.20 ± 1.754) and the 6400 mg/kg BW group (T3; 2.84 ± 0.698). The lowest IRS score was found in the negative control group (C−), which was not exposed to heat stress (1.28 ± 0.521).

Statistical analysis using the Mann–Whitney U test showed that most pairwise comparisons were significantly different ($p < 0.05$), as detailed in Table 2. Significant differences were observed between the positive control (C+) and all other groups, including T1 ($p = 0.028$), T2 ($p = 0.009$), T3 ($p = 0.009$), and C− ($p = 0.009$). Significant differences were also noted between T1 and T2 ($p = 0.047$), T1 and T3 ($p = 0.009$), and T1 and C− ($p = 0.009$). However, no significant difference was found between T2 and T3 ($p = 0.672$), suggesting a plateau in the response at higher doses. Representative IHC images illustrating the immunoreactivity of HSP-70 in testicular tissues are shown in Figure 1.

IL-6 Expression in Rooster Testes

Immunohistochemical analysis of IL-6 expression in rooster testicular tissues revealed significant differences among treatment groups, as indicated by the Kruskal–Wallis test ($p < 0.05$). Further pairwise comparisons were conducted using the Mann–Whitney U test to determine the specific intergroup differences.

As shown in Table 3, the highest IL-6 expression was recorded in the positive control group (C+), which was subjected to heat stress without treatment (9.08 ± 0.672). The group treated with 1600 mg/kg BW of *Cucurbita moschata* seed extract (T1) demonstrated a lower expression score (5.96 ± 1.722), while further reductions were observed in T2 (3200 mg/kg BW; 3.88 ± 0.944) and T3 (6400 mg/kg BW; 3.28 ± 1.035). The lowest IL-6 expression was found in the negative control group (C−), which was not exposed to heat stress (2.60 ± 0.860).

Results of the Mann–Whitney U test are detailed in Table 4. Significant differences (p <

Table 3. Mean IRS Scores of IL-6 Expression for Each Treatment

Treatment	IL-6 Expression Score (Mean ± SD)
C− (Negative Control)	2.60 ± 0.860
C+ (Positive Control)	9.08 ± 0.672
T1 (Pumpkin seed extract 1600 mg/kg BW)	5.96 ± 1.722
T2 (Pumpkin seed extract 3200 mg/kg BW)	3.88 ± 0.944
T3 (Pumpkin seed extract 6400 mg/kg BW)	3.28 ± 1.035

Table 4. Mann-Whitney Test Between Treatment Groups on IL-6 Expression

Group	Compared to	Sig (p-value)
C+	T1	0.046*
	T2	0.009*
	T3	0.009*
	C−	0.009*
T1	T2	0.026*
	T3	0.012*
	C−	0.009*
T2	T3	0.344
	C−	0.074
T3	C−	0.344

*Significant at p < 0.05

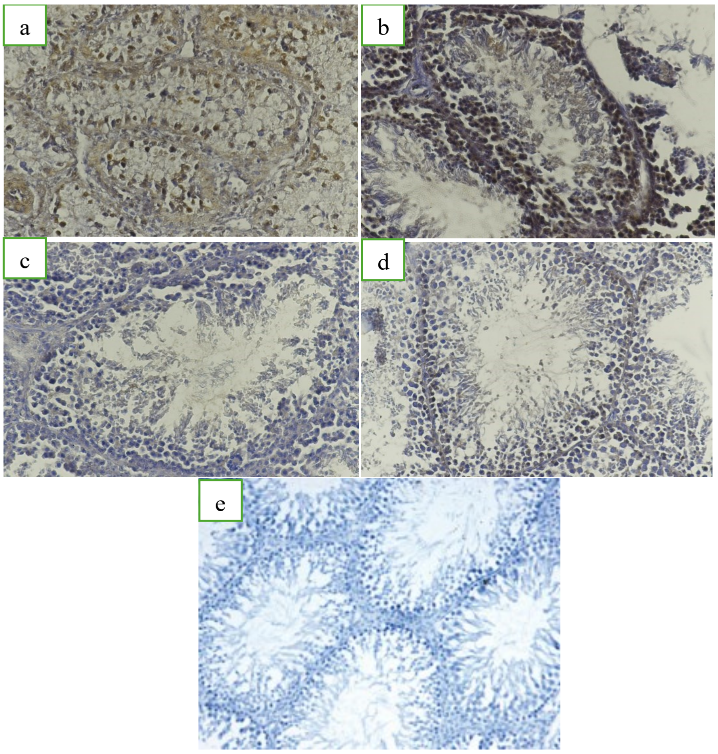

Figure 2. IL-6 expression in chicken testes a. C+ (positive control), b. T1 (pumpkin seed extract 1600 mg/BW), c. T2 (pumpkin seed extract 3200 mg/BW), d. (pumpkin seed extract 6400 mg/BW), e. Negative control (normal). (Nikon Eclipse Ci; 400×).

0.05) were found between the C+ group and all other groups, including T1 ($p = 0.046$), T2 ($p = 0.009$), T3 ($p = 0.009$), and C− ($p = 0.009$). T1 also showed significantly different expression compared to T2 ($p = 0.026$), T3 ($p = 0.012$), and C− ($p = 0.009$). However, no statistically significant difference was observed between T2 and T3 ($p = 0.344$), nor between T3 and C− ($p = 0.344$), indicating a plateau in IL-6 suppression at higher extract doses. Immunohistochemical staining patterns of IL-6 expression across groups are presented in Figure 2.

DISCUSSION

The presence of Heat Shock Protein 70 (HSP-70) in testicular tissue is widely recognized as a hallmark of cellular response to heat stress, highlighting both protein damage and the activation of protective cellular mechanisms. As a molecular chaperone, HSP-70 plays an essential role in helping proteins maintain their proper structure, especially under stress conditions. It works by preventing protein misfolding and aiding in the repait of damaged proteins (Wang *et al*., 2019; Shehata *et al.*, 2020). Because of this, elevated HSP-70 expression is generally used as a marker of cellular stress, particularly when triggered y elevated temperatures.

In our findings, the group of roosters exposed to heat stress without any treatment (positive control C^+) showed the highest average HSP-70 immunoreactive score (IRS), reaching 7.52±1.044. This clearly indicates a strong stress response at the cellular level. These results align with earlier reports that heat exposure significantly boosts HSP-70 expression in both avian and mammalian models (Wang *et al*., 2015). Interestingly, treatment with pumpkin seed (*Cucurbita moschata*) ~~extract~~ appeared to reduce HSP-70 expression in a dose dependent manner. A slight reduction was observed in the T1 group (1600 mg/ kg BW), while a more pronounced reduction was observed at higher doses, specifically in T2 (3200 mg/kg) and T3 (6400 mg/ kg).

This reduction suggests that *C. moschata* extract may provide a certain degree of protection against heat- induced oxidative stress.

It's likely that this defensive effect is due to the presence of natural antioxidants like flavonoids, carotenoids, and vitamin E, all of which are known to neutralize reactive oxygen species (Borecka and Karaś, 2025). By reducing oxidative pressure, cells appea to quire less HSP-70, which helps maintain internal stability and protein function (Zhou *et al*., 2017). Notably, the T3 group recorded HSP-70 levels nearly identical to the non-stressed control group (C^{-}), at 1.28 ± 0.521. This suggests that a high dose of extract may effectively suppress the cellular stress response and help protect testicular tissue from heat damage.

In addition to HSP-70, this study also found that Interleukin-6 (IL-6) expression rose significantly in the testes of heat-stressed roosters. IL-6 is well known for its role in immune regulation and tends to increase rapidly under conditions particularly during heat stress. The elevated IL-6 levels observed in the C^{+} group probably reflect a typical seditious response caused by prolonged heat exposure. These results are in line with Al-Zghoul *et al*. (2019), who reported that heat stress can spark the NF-κB signaling pathway, thereby increasing the production of IL-6 and other pro-inflammatory cytokines, and contributing to testicular tissue damage and impaired spermatogenesis.

Supplementation with *C. moschata* extract helped to significantly lower IL-6 levels, especially in the T2 and T3 groups. This points to the extract implicitanti-inflammatory action, probably due to its bioactive compounds, such as tocopherols and flavonoids, which are known to interfere with inflammatory signaling. Supporting this, Al-Okbi *et al.* (2017) have noted that species within the *Cucurbita rubric* contain composites that modulate cytokine production, while Bardaa *et al*. (2020) highlighted their effectiveness in combating oxidative stress-related conditions.

Importantly, the T3 group's IL-6 levels were not statistically different from those in the C^{-} group, suggesting that the highest extract dose capable of reversing most of the inflammatory effects of heat exposure. This result may indicate a restoration of immune homeostasis in the testes, potentially through mechanisms like membrane stabilization, antioxidant pathway activation, or repression of pro-inflammatory gene expression. However, the minimum difference observed between the T2 and T3 boluses hints at a implicit possible saturation point beyond which additional extract does not provide further benefit. This could be due to natural limits in tissue response or maybe reduced absorption efficiency at higher concentrations.

CONCLUSION

The *Cucurbita moschata* seed appears to hold potential as an anti-inflammatory agent for poultry under heat stress. Testicular tissue may be protected by the highest dose (6400 mg/kg BW), which significantly decreased IL-6 expression and brought it closer to levels of non-stressed controls. These findings highlight the potential of *C. moschata* in mitigating heat-induced reproductive damage. Clarifying its biological mechanisms and assessing its long-term effects on reproductive function under chronic heat stress requires more research.

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CONFLICT OF INTEREST

Authors declare no conflict of interest related to the publication of this manuscript.

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