Research Article

Fucoidan Nanoparticles Attenuate Heat Stress-Induced Testicular Injury in Rats Through Antioxidant and Anti-Inflammatory Effects

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Abstract | Heat stress is a major contributor to male reproductive dysfunction, primarily through oxidative damage, inflammatory activation, and disruption of testicular architecture. Fucoidan, a sulfated polysaccharide isolated from brown seaweed, possesses antioxidant and anti-inflammatory properties; however, its protective efficacy in nanoparticle form against heat-induced testicular injury remains unclear. This study investigated whether fucoidan nanoparticles protective testicular damage induced by heat stress in male Wistar rats. Fucoidan nanoparticles were prepared by high-energy ball milling and characterized by scanning electron microscopy and particle size analysis. Rats were exposed to 40 °C for 3 h/day for 14 consecutive days and treated orally with fucoidan nanoparticles at doses of 75, 150, or 300 mg/kg body weight for 18 days, 1 h before heat stress exposure. On day 18, testicular tissues were analyzed for malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and histopathological changes. Fucoidan nanoparticles showed irregular morphology with a rough surface and a mean particle size of 530.2 ± 38.27 nm. Heat stress markedly elevated MDA and pro-inflammatory cytokines, reduced SOD and GPx activities, and induced substantial histological damage in the testes. Among the tested doses, fucoidan nanoparticles at 300 mg/kg body weight produced the most pronounced protective effect, significantly reducing oxidative stress and inflammatory markers while restoring antioxidant enzyme activities and improving testicular histoarchitecture. Lower doses (75 and 150 mg/kg body weight) showed limited efficacy. Overall, fucoidan nanoparticles exerted dose-dependent testicular protection against heat stress, most likely through coordinated antioxidant and anti-inflammatory effects. These findings should be interpreted as preclinical evidence and require further validation through molecular, reproductive, pharmacokinetic, and safety studies before translational application can be considered.

Keywords | Fucoidan nanoparticles, Heat stress, Testicular injury, Oxidative stress, Inflammation

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INTRODUCTION

Heat stress is a major environmental challenge that disrupts physiological homeostasis in both humans and animals. It occurs when exposure to elevated ambient temperatures exceeds the body's thermoregulatory capacity, leading to cellular dysfunction, tissue injury, and organ damage (Abdollahi *et al.*, 2023; Shahat *et al.*, 2020;

Thanh *et al*., 2020). Among reproductive organs, the testes are particularly vulnerable to heat stress because normal spermatogenesis requires a temperature lower than core body temperature. Disruption of this thermal balance has been shown to impair sperm quality, alter testicular endocrine function, and ultimately reduce male fertility (El-Sherbiny *et al*., 2022; Halder *et al*., 2020).

The pathogenesis of heat stress-induced testicular injury is closely associated with oxidative stress and inflammatory responses (da Silva *et al*., 2024; Delkhosh *et al*., 2021). Excessive production of reactive oxygen species (ROS), including superoxide anion (O2-), hydroxyl radicals, and hydrogen peroxide (H_2O_2), can overwhelm endogenous antioxidant defense systems and promote oxidative injury in Sertoli cells, Leydig cells, and spermatogenic cells (Liu *et al*., 2022; Hafez *et al*., 2025; Sobanke *et al*., 2025). Heat stress may also affect antioxidant-related signaling, including nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates enzymes such as SOD, CAT, and GPx (Kurnijasanti *et al*., 2023a; Senturk and Ustundag, 2023; Wardani *et al*., 2023).

In addition to oxidative injury, heat stress activates inflammatory responses characterized by increased production of pro-inflammatory cytokines, particularly TNF-alpha and IL-6 (Hafez *et al*., 2025; Kurnijasanti *et al*., 2023; Wardani *et al*., 2022). These cytokines may amplify oxidative damage, promote lipid peroxidation, and disrupt the structural integrity of testicular tissue. MDA, a major end-product of lipid peroxidation, is widely used as a biomarker of oxidative membrane damage under heat stress conditions. Together, oxidative stress and inflammation can cause profound structural and functional impairment of the testes (Senturk and Ustundag, 2023; Hafez *et al*., 2025; Sobanke *et al*., 2025).

Given the central roles of oxidative stress and inflammation in heat-induced reproductive injury, increasing attention has been directed toward natural bioactive compounds as safer alternatives to conventional synthetic agents (El-Sherbiny *et al*., 2022; da Silva *et al*., 2024; Delkhosh *et al*., 2021; Wardani *et al*., 2023). Among these, fucoidan, a sulfated polysaccharide extracted from the cell walls of brown algae, has emerged as a promising candidate. Previous studies have demonstrated that fucoidan possesses antioxidant, anti-inflammatory, antidiabetic, immunomodulatory, and antitumor activities. Its protective actions are believed to involve enhancement of endogenous antioxidant defenses and suppression of pro-inflammatory cytokine expression (Kurnijasanti *et al*., 2024; Wardani *et al*., 2024; Wen *et al*., 2021).

Despite its pharmacological potential, the therapeutic application of conventional fucoidan may be limited by poor bioavailability, restricted tissue penetration, and insufficient stability under physiological conditions. Nanotechnology-based delivery systems have been proposed as an effective strategy to overcome these limitations (Sahu *et al*., 2021; Kurnijasanti *et al*., 2024). In the present context, reducing fucoidan into nanoparticle form may improve dispersion, cellular interaction, biological penetration, and overall pharmacological performance. This rationale supports the use of fucoidan nanoparticles as a potentially more efficient formulation for protecting testicular tissue against heat stress-induced oxidative and inflammatory injury.

Although the antioxidant and anti-inflammatory properties of fucoidan have been widely reported, evidence regarding the protective effects of fucoidan nanoparticles against heat stress-induced testicular injury remains limited. Therefore, the present study aimed to investigate the protective effects of fucoidan nanoparticles in a rat model of heat-induced testicular injury. Specifically, this study evaluated whether fucoidan nanoparticles attenuate oxidative stress, enhance antioxidant enzyme activity, suppress inflammatory responses, and improve testicular histoarchitecture. The novelty of this study lies in evaluating a nanoparticle formulation of fucoidan in a heat stress-induced testicular injury model and integrating biochemical and histopathological outcomes as preclinical evidence.

MATERIALS AND METHODS

Preparation of fucoidan nanoparticles

Fucoidan nanoparticles were prepared using a planetary ball-milling technique. Briefly, dried fucoidan powder was placed in a zirconia milling jar containing zirconia balls at a ball-to-powder ratio of 10:1. Milling was performed at 400 rpm in 15-min cycles with 5-min intervals to prevent overheating, resulting in a total effective milling time of 4 h. The milled powder was then passed through a 200-mesh stainless-steel sieve to remove large aggregates.

Particle size was analyzed by dynamic light scattering using a particle size analyzer. Morphological evaluation was performed by scanning electron microscopy (Wardani *et al*., 2023; Kurnijasanti *et al*., 2023).

Experimental animals

Male Wistar rats, aged 2.5-3 months and weighing approximately 200-250 g, were obtained from Gadjah Mada University, Yogyakarta, Indonesia. The animals were housed in plastic cages in an air-conditioned room maintained at 26 ± 2 °C under a 12-h light/dark cycle. Standard commercial laboratory chow and tap water were provided *ad libitum* throughout the experimental period.

Heat stress induction

Heat stress was induced by placing the rats in a heat chamber maintained at 40 °C for 3 h/day over 14 consecutive days. This protocol was selected because it reliably induces testicular injury without compromising animal survival. Heat exposure was initiated 1 h after oral administration of fucoidan nanoparticles or vehicle.

Experimental design

After acclimatization, rats were randomly allocated into five groups (n = 8 per group) using a simple randomization method to minimize selection bias. The experimental groups were as follows: (1) negative control (Vehicle): Rats received vehicle without heat stress exposure; (2) positive control (HS + Vehicle): Rats were exposed to heat stress and received vehicle; (3) HS + Fucoidan NP 75: rats were exposed to heat stress and treated with fucoidan nanoparticles at 75 mg/kg BW; (4) HS + Fucoidan NP 150: rats were exposed to heat stress and treated with fucoidan nanoparticles at 150 mg/kg BW; and (5) HS + Fucoidan NP 300: Rats were exposed to heat stress and treated with fucoidan nanoparticles at 300 mg/kg BW.

Fucoidan nanoparticles were administered orally by gavage once daily for 18 consecutive days, 1 h before heat stress exposure. The control groups received the corresponding vehicle according to the same treatment schedule. On day 18, all rats were anesthetized with diethyl ether, and the testes were harvested for biochemical and histopathological analyses. Testicular tissues were used for biochemical analysis, including determination of MDA, SOD, GPx, IL-6, and TNF-alpha, and for histopathological analysis using hematoxylin-eosin staining.

Measurement of MDA levels in testis tissue

Testis tissues were homogenized in cold phosphate-buffered saline, and the supernatant was collected after centrifugation. Lipid peroxidation was determined by quantifying malondialdehyde (MDA) levels using the thiobarbituric acid (TBA) reaction, which forms an MDA–TBA adduct measurable by spectrophotometry. The absorbance of the complex was recorded at 532 nm. To ensure sensitivity and reproducibility, a commercial Lipid Peroxidation Colorimetric/Fluorometric Assay Kit (Biovision K739-100, Milpitas, CA, USA) was used. MDA concentrations were normalized to tissue weight and expressed as nanomoles per milligram of tissue (nmol/mg tissue).

measurement of SOD and GPx levels in testis tissue

Approximately 50 mg of testis tissue was washed repeatedly with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) until clean. The sample was homogenized in 0.5 mL of sample buffer and centrifuged at 10,000 rpm for 10 min at 4 °C. The resulting supernatant was collected for analysis.

Superoxide dismutase (SOD) levels were determined using a rat SOD ELISA kit (Biovision K335-100, Milpitas, CA, USA), while glutathione peroxidase (GPx) levels were measured using a rat GPx ELISA kit (Biovision K762-100, Milpitas, CA, USA), according to the manufacturer's instructions. Briefly, samples were incubated in microplates at 37 °C for 90 min, followed by incubation with biotinylated antibodies for 60 min at 37 °C. After washing with PBS (0.01 M), the plates were incubated with avidin–biotin complex solution for 30 min and then developed with TMB substrate for 30 min at 37 °C. The reaction was terminated with stop solution, and absorbance was measured at 450 nm using a microplate reader. Enzyme concentrations were calculated from the standard curve and expressed as pg/mL.

Measurement of IL-6 and TNF-α levels in testis tissue

Testicular IL-6 and TNF-α concentrations were quantified using Rat IL-6 ELISA Kit (BMS625, Invitrogen, Thermo Fisher Scientific, USA) and Rat TNF-α ELISA Kit (KRC3012, Invitrogen, Thermo Fisher Scientific, USA), respectively, following the manufacturer's instructions. Briefly, a standard curve was generated using serially diluted recombinant standards. Testis tissue homogenate samples were diluted in assay diluent to fall within the dynamic range of the assay and assayed in duplicate. After incubation and washing steps, wells were treated sequentially with biotinylated detection antibody and streptavidin–HRP, and then developed using TMB substrate solution. The enzymatic reaction was terminated with 1 N H_2SO_4, and absorbance was measured at 450 nm with wavelength correction at 570–620 nm using a microplate reader.

Histopathological evaluation of testis tissue

Testicular tissue samples were fixed in 10% neutral-buffered formalin at room temperature for 48 hours, followed by standard dehydration and paraffin embedding procedures. Paraffin-embedded blocks were sectioned at a thickness of 4 µm using a rotary microtome. The tissue sections were deparaffinized in xylene for 10 minutes at room temperature and subsequently rehydrated through a graded ethanol series. Hematoxylin and eosin (H & E) staining was performed for 10 minutes at room temperature to visualize tissue architecture. Finally, histological evaluation was conducted under a light microscope (Nikon Eclipse 80i; Nikon Corporation).

Histopathological damage of testicular tissue was semi-quantitatively assessed using the Cosentino classification.

Score 1 indicated normal testicular architecture with orderly arranged germinal cells; Score 2 indicated less orderly germinal cells and closely packed seminiferous tubules; Score 3 indicated disorganized germinal cells with shrunken pyknotic nuclei and less distinct seminiferous tubule borders; and Stage 4 indicated closely packed seminiferous tubules with coagulative necrosis of germinal cells.

Statistical analysis

Data were expressed as mean +/- standard deviation (SD). Statistical analysis was performed using SPSS software. Data normality was assessed using the Shapiro-Wilk test, and homogeneity of variance was evaluated using Levene's test. Differences among groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons when parametric assumptions were met. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Characterization of fucoidan nanoparticles

Scanning electron microscopy showed that the fucoidan nanoparticles had irregular morphology with a rough surface. Dynamic light scattering demonstrated a mean particle size of 530.2 ± 38.27 nm (Figure 1).

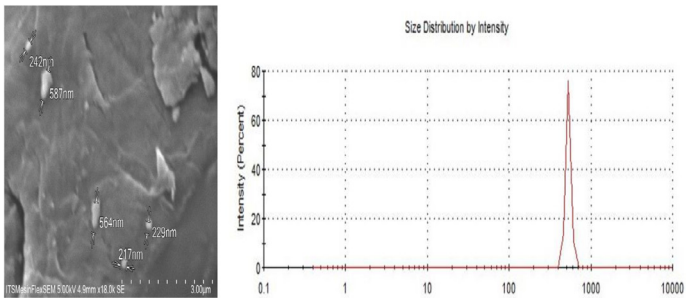

Figure 1: Characterization of fucoidan nanoparticles. Scanning electron microscopy image of fucoidan nanoparticles is shown in panel (A), and particle size distribution determined by dynamic light scattering is shown in panel (B).

Effect of fucoidan nanoparticles on testicular MDA levels under heat stress

MDA levels were markedly elevated in the heat stress group compared to the control, indicating enhanced lipid peroxidation and oxidative stress in testicular tissue. Treatment with fucoidan nanoparticles at 300 mg/kg BW significantly reduced MDA concentrations ($p < 0.05$), with values approaching those observed in the control group. In contrast, doses of 75 and 150 mg/kg BW did not result in significant reductions compared to the heat stress group (Table 1).

Table 1: Effect of fucoidan nanoparticles on testicular MDA levels in heat stress rats.

Group	Means ± SD
	MDA (nmol/mg)
Control group	3.1^{a} ± 0.3
Heat Stress	6.8^{b} ± 0.8
Fucoidan Nano 75 mg/kg BW	6.5^{b} ± 0.6
Fucoidan Nano150 mg/kg BW	6.2^{b} ± 0.4
Fucoidan Nano 300 mg/kg BW	4.5^{c} ± 0.2

In the same column, different superscripts indicate the significant difference between means ($p < 0.05$).

Eefect of fucoidan nanoparticles on SOD and GPx levels in testicular tissue under heat stress

Rats exposed to heat stress exhibited significant decreases in the activities of SOD, and GPx, compared to the control group, indicating compromised testicular antioxidant defense mechanisms. Administration of fucoidan nanoparticles at 300 mg/kg BW significantly restored the activity of these enzymes ($p < 0.05$). In contrast, groups receiving 75 and 150 mg/kg BW showed slight but not statistically significant increases in antioxidant enzyme activity compared to the heat stress group (Table 2).

Table 2: Effects of fucoidan nanoparticles on testicular SOD and GPx levels in heat stress rats.

Group	Mean ± SD	
	SOD (U/mg protein)	GPx (nmol/mg protein)
Control	15.8^{a} ± 1.1	12.6^{a} ± 0.7
Heat stress	9.2^{b} ± 0.9	6.4^{b} ± 0.9
Fucoidan Nano 75 mg/kg BW	9.8^{b} ± 0.8	6.7^{b} ± 0.8
Fucoidan Nano 150 mg/kg BW	10.5^{b} ± 0.7	7.1^{b} ± 0.7
Fucoidan Nano 300 mg/kg BW	12.1^{c} ± 0.9	9.6^{c} ± 0.5

In the same column, different superscripts indicate the significant difference between means ($p < 0.05$).

Effect of fucoidan nanoparticles on TN-α and IL-6 levels in testicular tissue under heat stress

Levels of pro-inflammatory cytokines TNF-α and IL-6 were markedly elevated in the heat stress group compared to the control, indicating induction of inflammatory responses in testicular tissue. Administration of fucoidan nanoparticles at 300 mg/kg BW significantly reduced these cytokine (TNF-α and IL-6) levels ($p < 0.05$), whereas treatment with 75 and 150 mg/kg BW did not produce significant changes relative to the heat stress group (Table 3).

Effect of fucoidan nanoparticles histological of rat testis under heat stress

Histological evaluation of rat testicular tissue is presented

in Figure 2. In the control group, the seminiferous tubules exhibited normal morphology with a regular arrangement of germinal epithelium and well-organized spermatogenic cells. In contrast, rats exposed to heat stress demonstrated pronounced pathological changes, including seminiferous tubule atrophy, thinning of the germinal epithelium, and a marked reduction in spermatogenic cell populations. Administration of fucoidan nanoparticles at doses of 75 mg/kg BW and 150 mg/kg BW resulted in only mild histological alterations, suggesting partial protection against heat stress–induced testicular damage. Notably, treatment with fucoidan nanoparticles at the highest dose of 300 mg/kg BW markedly preserved testicular architecture, maintaining seminiferous tubule morphology and cellular organization close to normal. These findings indicate that fucoidan nanoparticles provide a dose-dependent protective effect against heat stress–induced testicular injury.

Heat stress markedly increased the Cosentino histological damage score compared with the control group, indicating moderate to severe testicular injury. Treatment with fucoidan nanoparticles reduced the histological damage score in a dose-dependent manner, with the 300 mg/kg BW group showing the greatest improvement in testicular architecture (Table 4).

Table 3: Effect of fucoidan nanoparticle on testicular tissue of IL-6 and TNF-α levels in heat stress rats.

Group	Mean ± SD	
	IL-6 (pg/ml)	TNF-α (pg/ml)
Control	20.5 [a] ± 2.8	24.4 [a] ± 2.5
Heat Stress	46.7 [b] ± 4.2	55.2 [b] ± 5.6
Fucoidan nano 75 mg/kg BW	42.3 [b] ± 3.6	53.6 [b] ± 5.2
Fucoidan nano 150 mg/kg BW	36.8 [b] ± 3.9	45.1 [b] ± 4.7
Fucoidan nano 300 mg/kg BW	26.7 [c] ± 2.4	30.9 [c] ± 2.6

In the same column, different superscripts indicate the significant difference between means ($p < 0.05$).

Table 4: Effect of fucoidan nanoparticles on histological damage score of rat testis under heat stress.

Group	Means ± SD
	Histological damage score
Control group	1.02 [a] ± 0.12[a]
Heat Stress	3.50 [b] ± 0.53c
Fucoidan Nano 75 mg/kg BW	3.20 [b] ± 0.41c
Fucoidan Nano150 mg/kg BW	2.50 [c] ± 0.17[b]
Fucoidan Nano 300 mg/kg BW	1.76 [d] ± 0.12[b]

In the same column, different superscripts indicate the significant difference between means ($p < 0.05$).

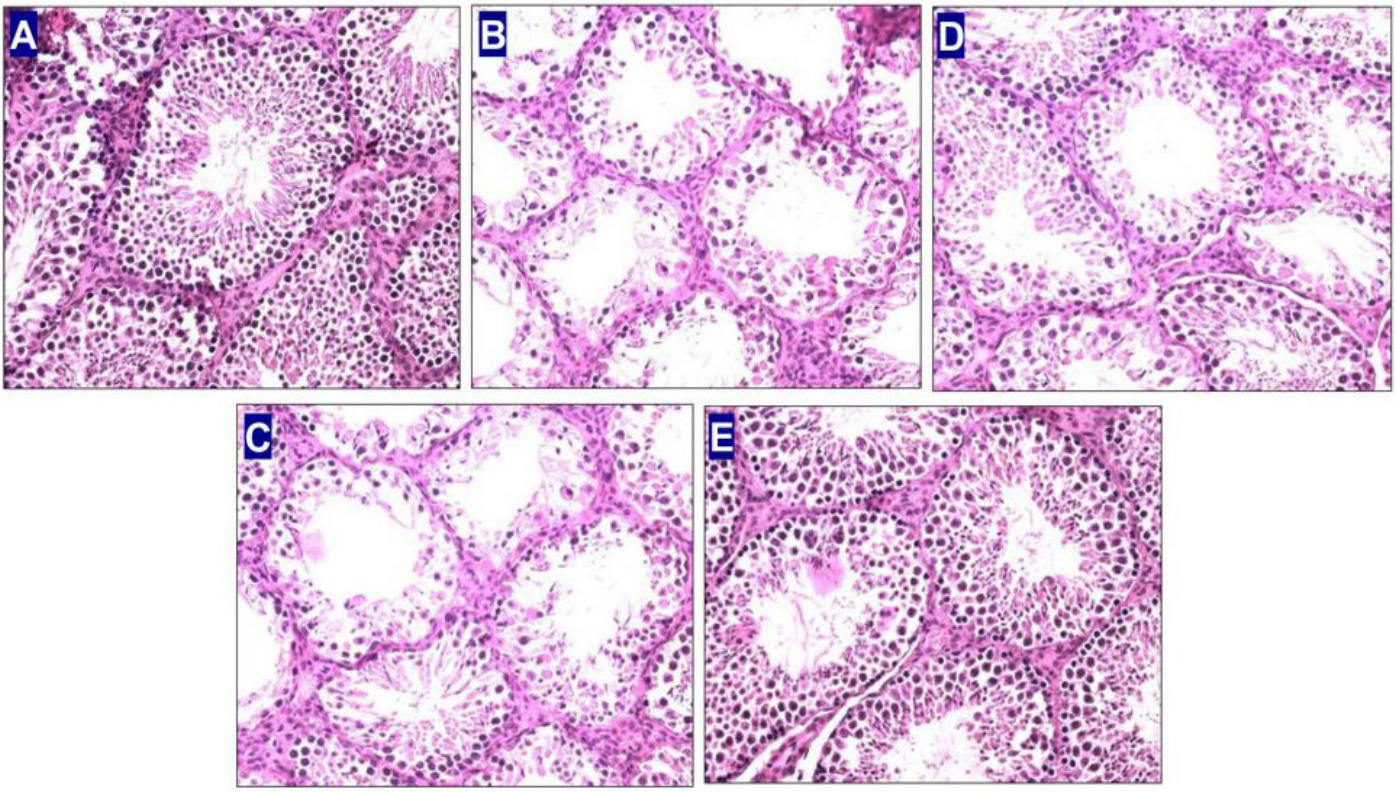

Figure 2: Testis Tissue Histology in Rats. The testis cells of control rats displayed a regular morphology (A). Hat stress rats exhibited signs of seminiferous tubule atrophy, thinning of the germinal epithelium, and a reduced number of spermatogenic cells; (B). Administration of fucoidan nanoparticles at a dose of 75 mg/kg BW (C) and 150 mg/kg BW (D) showed mild testicular tissue damage, while a dose of 300 mg/kg can inhibit testicular tissue damage(E). Magnification: 400x, H&E staining.

DISCUSION

This study demonstrated that repeated exposure to heat stress (40 °C, 3 h/day for 14 consecutive days) induced substantial testicular injury in Wistar rats, as reflected by increased lipid peroxidation, reduced antioxidant enzyme activity, elevated pro-inflammatory cytokines, and marked histopathological alterations (da Silva *et al*., 2024; Delkhosh *et al*., 2021). These results reinforce previous evidence that heat stress disrupts testicular homeostasis through interconnected oxidative and inflammatory mechanisms (El-Sherbiny *et al*., 2022; da Silva *et al*., 2024; Delkhosh *et al*., 2021). The testes are highly susceptible to thermal injury because spermatogenesis depends on a tightly regulated microenvironment, and even moderate increases in temperature may impair cellular integrity and reproductive function.

Among the tested doses, fucoidan nanoparticles at 300 mg/kg body weight provided the most pronounced protection against heat stress-induced injury. This effect was evidenced by lower MDA levels, restoration of SOD and GPx activities, reduction of TNF-alpha and IL-6 levels, and improvement of testicular histoarchitecture. In contrast, the lower doses of 75 and 150 mg/kg body weight were not sufficient to produce significant protection for most measured parameters, suggesting a dose-dependent protective effect. This dose-response pattern may indicate that the severity of oxidative and inflammatory damage induced by heat stress requires a higher concentration of fucoidan nanoparticles to achieve measurable biological efficacy.

The antioxidant effect of fucoidan observed in this study is consistent with previous reports describing its capacity to scavenge reactive oxygen species and enhance endogenous antioxidant defense systems. Fucoidan may exert these effects both directly, through its sulfate-rich molecular structure, and indirectly, by influencing redox-sensitive signaling pathways. Previous studies suggest that Nrf2 may contribute to the regulation of antioxidant enzymes, including SOD and GPx (Wardani *et al*., 2022, 2024). However, because Nrf2 expression or activation was not directly measured in the present study, this mechanism should be interpreted cautiously.

The anti-inflammatory effect of fucoidan nanoparticles was evident from the reduced TNF-alpha and IL-6 levels in treated animals. These cytokines play central roles in heat stress-associated inflammatory responses and contribute to cellular degeneration and structural disruption in testicular tissue. Fucoidan has been suggested to inhibit inflammatory signaling pathways, including NF-kappaB activation, thereby limiting the expression of pro-inflammatory mediators. Nevertheless, NF-kappaB signaling was not directly examined in this study; therefore, its involvement remains a plausible explanation that requires further molecular confirmation (Wardani *et al*., 2022, 2023; Liu *et al*., 2022).

Importantly, the histopathological observations were consistent with the biochemical findings. Heat stress caused degeneration of seminiferous tubules, germ cell depletion, and disruption of testicular architecture, whereas fucoidan nanoparticles at 300 mg/kg body weight markedly alleviated these structural abnormalities. Treatment with fucoidan nanoparticles reduced the histological damage score in a dose-dependent manner, with the 300 mg/kg BW group showing the greatest improvement in testicular architecture

The findings may have broader implications for male reproductive health in populations exposed to chronic environmental, occupational, or climate-related heat stress. In veterinary and biomedical contexts, natural marine-derived bioactive compounds delivered through nanotechnology may represent an area of interest for supportive or preventive strategies. However, the present findings remain preclinical and should not be interpreted as direct evidence of clinical efficacy.

Several limitations should be acknowledged. First, although the present findings demonstrate antioxidant, anti-inflammatory, and histological protective effects of fucoidan nanoparticles, specific molecular pathways such as Nrf2 and NF-kappaB were not directly measured. Second, sperm quality, reproductive hormone concentrations, mating performance, and fertility outcomes were not evaluated. Third, detailed pharmacokinetic, biodistribution, and long-term safety studies are required to confirm the absorption, tissue distribution, and safety profile of fucoidan nanoparticles. Fourth, quantitative histological scoring and complete nanoparticle characterization should be reported to strengthen reproducibility and interpretation. Therefore, additional preclinical studies are necessary before translational application can be considered.

CONCLUSION

In conclusion, fucoidan nanoparticles, particularly at 300 mg/kg BW, attenuated heat stress-induced testicular injury in rats by reducing oxidative stress, improving antioxidant enzyme activity, suppressing inflammatory cytokines, and preserving testicular histoarchitecture. These findings suggest that fucoidan nanoparticles may serve as a promising preclinical candidate for further investigation in heat stress-associated male reproductive impairment. However, additional studies evaluating molecular signaling pathways, sperm quality, reproductive hormones, fertility

outcomes, pharmacokinetics, and long-term safety are required before translational application can be established

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NOVELTY STATEMENT

This study provides novel preclinical evidence that fucoidan nanoparticles attenuate heat stress-induced testicular injury through coordinated antioxidant and anti-inflammatory mechanisms. By integrating biochemical and histopathological evaluations, this work expands current knowledge regarding the therapeutic potential of nanostructured fucoidan for protecting male reproductive tissues under thermal stress.

AUTHOR'S CONTRIBUTION

Conceptualization: Siti Nurfitria, Rochmah Kurnijasanti, Sri Agus Sudjarwo, Mohammad Rais Mustafa; Methodology: Rochmah kurnijasanti, Siti Nurfitria, Sri Agus Sudjarwo; Formal analysis and investigation: Siti Nurfitria, Giftania Wardani, Rochmah kurnijasanti; Writing - original draft preparation: Sri Agus Sudjarwo, Siti Nurfitria; Writing - review and editing: Sri Agus Sudjarwo, Mohammad Rais Mustafa; Funding acquisition: Sri Agus Sudjarwo; Resources: Sri Agus Sudjarwo, Giftania Wardani; Supervision: Sri Agus Sudjarwo

Ethical approval

All experimental procedures were conducted in accordance with established guidelines for the care and use of laboratory animals and were approved by the Animal Research Ethics Committee, Faculty of Veterinary Medicine, Airlangga University, Indonesia (Ethical Approval No. 76/FKH.UA/7/2025).

Generative AI and AI-assisted technology

Generative AI and AI-assisted technologies were used only to improve language clarity, grammar, and readability of the manuscript. The authors take full responsibility for the scientific content, data interpretation, and conclusions presented in this work.

Conflict of interest

The authors declare that there are no financial or personal relationships that could be viewed as potential conflicts of interest concerning this publication.

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