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Original Article

Protective effects of black garlic extract on sperm quality and liver histopathology following cigarette smoke exposure: an in vivo experimental study

Lisdiana [1]✉, Nurul Amida [1], Dwi Susanti [1], Djuna Lamondo [2], Annisa Basar [3]

[1] *Department of Biology Education, Faculty of Science and Mathematics, Universitas Negeri Semarang, Semarang, Central Java, Indonesia*
[2] *Department of Biology Education, Faculty of Science and Mathematics, Universitas Negeri Gorontalo, Gorontalo, Indonesia*
[3] *Department of Educational and Foundations Humanities, Faculty of Education, Universiti Malaya, Kuala Lumpur, Malaysia*

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CORRESPONDENCE

Phone: +62 857 1324 3920

E-mail: Lisdiana@unnes.mail.id

A B S T R A C T

Background: Cigarette smoke contains numerous toxic compounds that generate reactive oxygen species, leading to oxidative stress that can impair reproductive function and induce liver damage. Natural antioxidants such as black garlic have been reported to possess strong free radical–scavenging properties.

Purpose: This study aimed to evaluate the protective effects of black garlic extract on sperm quality, liver enzyme levels, and liver histopathology in rats exposed to cigarette smoke.

Methods: A randomized post-test-only control group experimental design was used. Twenty-five male Wistar rats were divided into five groups: normal control, cigarette smoke exposure, and three treatment groups receiving black garlic extract at doses of 250, 500, and 1000 mg/kg body weight for 30 days. Sperm concentration, viability, and motility were assessed. Liver function was evaluated using serum ALT and AST levels, and liver histopathology was examined using hematoxylin–eosin staining. Data were analyzed using one-way ANOVA followed by post hoc tests.

Results: Cigarette smoke exposure significantly reduced sperm concentration, viability, and motility and induced hepatocellular degeneration and necrosis. Administration of black garlic extract improved sperm parameters and reduced histopathological liver damage, with the most pronounced protective effect observed at a dose of 500 mg/kg body weight. However, no significant differences were observed in serum ALT and AST levels among groups.

Conclusion: Black garlic extract exerts protective effects against cigarette smoke–induced reproductive and hepatic damage, with the most effective dose observed at 500 mg/kg body weight. These findings support its potential as a natural antioxidant for mitigating oxidative damage. Further studies are required to elucidate the underlying molecular mechanisms and identify the active compounds responsible for these effects.

INTRODUCTION

Cigarette smoking remains a major global public health problem and is one of the leading causes of preventable morbidity and mortality worldwide. In Indonesia, tobacco consumption continues to be particularly high. According to the World Health Organization, approximately 225,700 deaths occur annually in Indonesia due to smoking and tobacco-related diseases. National surveys conducted in 2013 and 2018 also reported that tobacco use remains prevalent among adults and has increased among adolescents aged 10–18 years.[1,2] Cigarette smoke contains thousands of harmful chemicals, including nicotine, tar, carbon monoxide, benzene, hydrogen cyanide, hydrogen peroxide, superoxide, acrolein, nitrogen oxides, ammonia, ethanol, polycyclic aromatic hydrocarbons, radioactive substances, and heavy metals. Many of these compounds act as carcinogens and major sources of free radicals that induce oxidative stress in biological systems.[3-6]

Oxidative stress resulting from excessive production of reactive oxygen species (ROS) is known to impair male reproductive function. Nicotine and other toxic components

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of cigarette smoke have been shown to negatively affect spermatozoa quality, including concentration, viability, and motility.[7] ROS can damage sperm membranes through lipid peroxidation, disrupt DNA integrity, and trigger apoptosis in spermatozoa, ultimately reducing male fertility potential.[6,8] These mechanisms highlight the detrimental impact of cigarette smoke on reproductive health.

In addition to reproductive impairment, cigarette smoke–induced oxidative stress can damage other vital organs, particularly the liver. As the primary organ responsible for detoxification, the liver is highly susceptible to oxidative injury caused by toxic compounds in cigarette smoke. Excessive free radical production disrupts redox balance within hepatocytes, leading to cellular damage, inflammation, and necrosis.[9] When hepatocytes are damaged, intracellular enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are released into the bloodstream, making them important biomarkers of liver injury.[10] Therefore, cigarette smoke exposure may simultaneously compromise reproductive function and hepatic integrity through oxidative stress–mediated mechanisms.

Antioxidants play an essential role in neutralizing free radicals and protecting cells from oxidative damage. Although endogenous antioxidant systems exist, they are often insufficient under conditions of excessive oxidative stress. Consequently, exogenous antioxidants from natural sources may provide additional protective effects. Black garlic, produced through controlled fermentation of fresh garlic, has gained attention due to its enhanced antioxidant properties. This process increases the levels of bioactive compounds such as polyphenols, flavonoids, and S-allyl cysteine (SAC), which exhibit strong free radical–scavenging activity.[11,12]

Previous studies have demonstrated the antioxidant and protective properties of black garlic in various experimental models. However, most studies have focused on its effects on single physiological systems. Evidence regarding its simultaneous protective effects on both reproductive parameters and liver function under cigarette smoke exposure remains limited and insufficiently explored. Therefore, this study aimed to evaluate the protective effects of black garlic extract on sperm quality, liver enzyme levels (ALT and AST), and liver histopathology in male Wistar rats exposed to cigarette smoke. By integrating reproductive and hepatic outcomes within a single experimental model, this study provides a more comprehensive evaluation of the potential protective role of black garlic against cigarette smoke–induced oxidative damage.

METHOD

Study Design

This study employed a true experimental design using a randomized post-test-only control group approach.

Study Setting

The study was conducted from February to July 2021 at multiple laboratories in Central Java and Yogyakarta, Indonesia. Animal experiments were performed at the Animal Maintenance and Intervention Facility, Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang. Histopathological examination was conducted at the Veterinary Center Wates, Yogyakarta. Serum ALT and AST analyses were performed at the Central Java Provincial Health Laboratory and Medical Device Testing Center, with additional analyses conducted at the Waspada Diagnostic Laboratory, Semarang.

Materials

The equipment included a smoking chamber, nebulizer, microcentrifuge, digital microscope, analytical balance, oven, micropipettes, and standard laboratory tools for tissue and biochemical analysis. The materials consisted of male Wistar rats (Rattus norvegicus), black garlic extract prepared using 96% ethanol, unfiltered kretek cigarettes, standard rat pellets, 10% neutral buffered formalin, reagents for ALT and AST assays, and materials for sperm analysis including slides, cover glasses, staining reagents, and physiological saline.

Preparation of Black Garlic Extract

Black garlic extract was prepared using a maceration method with 96% ethanol. A total of 440 g of dried black garlic was ground into powder and macerated in 2.2 L of ethanol for five days with daily stirring. The filtrate was concentrated to obtain a thick extract, which was then dissolved in 600 mL of distilled water to prepare a stock solution. The extract was administered at doses of 250, 500, and 1000 mg/kg body weight.

In Vivo Procedures

Animal Preparation

Twenty-five male Wistar rats (Rattus norvegicus), aged 2–3 months and weighing 180–200 g, were used. Animals were acclimatized for seven days under controlled conditions with free access to standard feed and water.

Experiments Procedure

Animals were randomly divided into five groups (n = 5 per group): P0 (normal control), P1 (cigarette smoke exposure), P2 (smoke + black garlic 250 mg/kgBW), P3 (smoke + black garlic 500 mg/kgBW), and P4 (smoke + black garlic 1000 mg/kgBW). Cigarette smoke exposure was conducted using a smoking chamber with exposure equivalent to two unfiltered kretek cigarettes per day for 30 days, with each session lasting approximately 30 minutes. At the end of the experiment (day 31), rats were anesthetized and sacrificed. Blood, liver tissue, and reproductive organs were collected for biochemical and histological analyses. Environmental conditions were maintained at 22–25 °C with a 12-hour light/dark cycle.

Sperm Quality Assessment

Spermatozoa were collected from the epididymis and vas deferens and diluted with physiological saline. Sperm concentration was measured using a hemocytometer. Viability was assessed using eosin–nigrosin staining, and motility was evaluated according to WHO criteria adapted for rodent models.

Analysis of Liver Enzymes

Serum ALT and AST levels were measured using the IFCC method without pyridoxal phosphate using DiaSys reagent kits.

Liver Histopathology

Liver tissues were fixed in 10% NBF, processed using standard histological techniques, and stained with hematoxylin–eosin. Sections were examined at 400× magnification across five microscopic fields. Histopathological scoring followed the Manja Roenigk method, evaluating parenchymatous degeneration, hydropic degeneration, and necrosis. Observations were conducted in a blinded manner.

Data Analysis

Data were expressed as mean ± SD. Normality was assessed using the Shapiro–Wilk test and homogeneity using Levene's test. Differences among groups were analyzed using one-way ANOVA followed by LSD or Duncan post hoc tests. Statistical analysis was performed using IBM SPSS Statistics version 27, with $p < 0.05$ considered significant.

Ethical Considerations

All procedures were approved by the Ethics Committee of the Faculty of Medicine, Universitas Negeri Semarang (No. 028/KEPK/EC/2021).

RESULTS

Sperm Quality Parameters

After 30 days of treatment, sperm concentration, viability, and progressive motility differed significantly among groups ($p < 0.05$) (Table 1). Rats exposed to cigarette smoke (P1) showed markedly reduced sperm concentration (18.0 ± 6.32 ×10⁶ cells/mL), viability (0.46 ± 0.33), and motility (30.40 ± 2.61%) compared with the normal control group (P0: 62.8 ± 7.82; 1.88 ± 0.09; 55.60 ± 4.56%, respectively). Administration of black garlic extract improved sperm quality in a dose-dependent manner. The 500 mg/kg body weight group (P3) demonstrated the most pronounced improvement, with sperm concentration (68.0 ± 9.79 ×10⁶ cells/mL) and motility (52.80 ± 6.10%) approaching normal control values. The 250 mg/kg group (P2) showed moderate improvement, whereas the 1000 mg/kg group (P4) exhibited partial recovery but did not surpass the effect observed at 500 mg/kg.

Liver Enzyme Levels (ALT and AST)

Serum ALT levels did not differ significantly among groups ($p > 0.05$) (Table 2). In contrast, AST levels were elevated in the cigarette smoke group (P1: 274.77 ± 51.07 U/L) compared with the normal control group (P0: 161.63 ± 46.58 U/L), indicating hepatic stress. Administration of black garlic extract at doses of 250 mg/kg (P2) and 500 mg/kg (P3) did not significantly reduce AST levels compared with the smoke-exposed group. However, the 1000 mg/kg group (P4) showed lower AST levels (185.43 ± 29.77 U/L), approaching control values. These findings suggest that short-term administration of black garlic extract had limited effects on biochemical liver markers.

Table 1. Sperm Quality Parameters in Rats Exposed to Cigarette Smoke and Treated with Black Garlic Extract for 30 Days (mean ± SD, n = 5 per group)

Group	Sperm Concentration (×10⁶ cells/mL)	Viability (%)	Progressive Motility (%)
P0	62.8 ± 7.82^{c}	1.88 ± 0.09^{c}	55.60 ± 4.56^{c}
P1	18.0 ± 6.32^{a}	0.46 ± 0.33^{a}	30.40 ± 2.61^{a}
P2	39.2 ± 5.93^{b}	1.25 ± 0.16^{b}	43.20 ± 2.28^{b}
P3	68.0 ± 9.79^{c}	1.45 ± 0.17^{b}	52.80 ± 6.10^{c}
P4	48.8 ± 6.09^{b}	1.39 ± 0.21^{b}	46.80 ± 3.63^{b}

Note: Different superscript letters within the same column indicate statistically significant differences ($p < 0.05$).P0 – Standard feed; P1 – Cigarette smoke; P2 – Smoke + Black garlic 250 mg/kg BW; P3 – Smoke + Black garlic 500 mg/kg BW; P4 – Smoke + Black garlic 1000 mg/kg BW

Table 2. Serum ALT and AST Levels in Rats Exposed to Cigarette Smoke and Treated with Black Garlic Extract for 30 Days (mean ± SD, n = 5 per group).

Group	ALT (U/L)	AST (U/L)
P0	127.83 ± 23.24^{a}	161.63 ± 46.58^{a}
P1	144.02 ± 27.88^{a}	274.77 ± 51.07^{bc}
P2	131.91 ± 22.16^{a}	307.34 ± 98.76^{c}
P3	142.73 ± 44.73^{a}	284.21 ± 54.78^{c}
P4	135.16 ± 27.38^{a}	185.43 ± 29.77^{ab}

Note: Different superscript letters indicate statistically significant differences ($p < 0.05$). P0 – Standard feed; P1 – Cigarette smoke; P2 – Smoke + Black garlic 250 mg/kg BW; P3 – Smoke + Black garlic 500 mg/kg BW; P4 – Smoke + Black garlic 1000 mg/kg BW

Liver Histopathology

Microscopic examination revealed clear differences in hepatocyte morphology among groups (Figure 1; Table 3). The normal control group (P0) exhibited intact hepatic architecture with regularly arranged hepatocytes and no evidence of degeneration or necrosis. In contrast, the cigarette smoke group (P1) showed marked liver damage, including parenchymatous degeneration (8.56 ± 0.98), hydropic degeneration (8.76 ± 1.12), and necrosis (2.32 ± 1.01).

Treatment with black garlic extract reduced the severity of these alterations. The 250 mg/kg group (P2) showed reduced necrosis but persistent degeneration, while the 500

mg/kg group (P3) demonstrated the most favorable histological profile, with improved hepatocyte organization, reduced degeneration, and absence of necrosis. In the 1000 mg/kg group (P4), mixed findings were observed, including reduced parenchymatous degeneration (6.44 ± 3.17) but increased necrosis (4.40 ± 2.22), indicating a less consistent protective effect. Overall, cigarette smoke exposure induced significant liver damage, while black garlic extract attenuated histopathological alterations, with the most optimal effect observed at a dose of 500 mg/kg body weight.

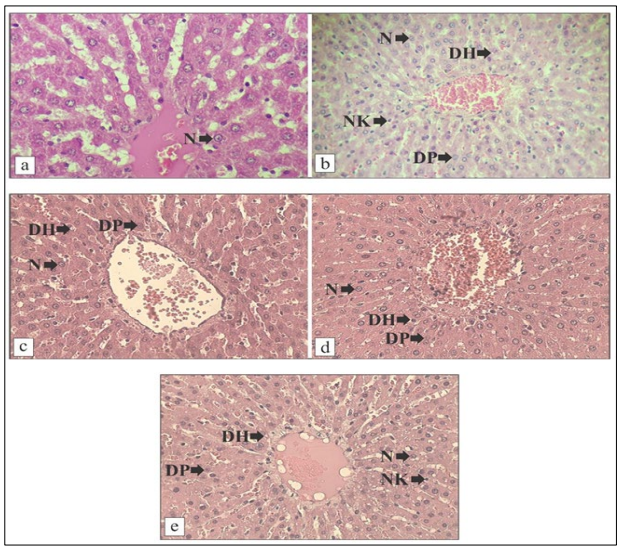

Figure 1. Representative histopathological images of rat liver tissue (hematoxylin–eosin staining, 400× magnification). (A) Normal control; (B) cigarette smoke exposure showing parenchymatous degeneration (DP), hydropic degeneration (DH), and necrosis (NK); (C) smoke + black garlic 250 mg/kgBW; (D) smoke + black garlic 500 mg/kgBW; (E) smoke + black garlic 1000 mg/kgBW.

Table 3. Liver Histopathological Damage Scores in Rats Exposed to Cigarette Smoke and Treated with Black Garlic Extract for 30 Days (mean ± SD, n = 5 per group).

Group	Normal Cells	Parenchymatous Degeneration	Hydropic Degeneration	Necrosis
P0	20.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
P1	0.36 ± 0.26	8.56 ± 0.98	8.76 ± 1.12	2.32 ± 1.01
P2	0.72 ± 0.22	11.28 ± 1.09	8.00 ± 1.26	0.00 ± 0.00
P3	1.00 ± 0.87	11.40 ± 3.84	7.60 ± 3.64	0.00 ± 0.00
P4	0.16 ± 0.26	6.44 ± 3.17	9.00 ± 1.67	4.40 ± 2.22

Note: Scores represent the mean number of affected cells observed across five microscopic fields.P0 – Standard feed; P1 – Cigarette smoke; P2 – Smoke + Black garlic 250 mg/kg BW; P3 – Smoke + Black garlic 500 mg/kg BW; P4 – Smoke + Black garlic 1000 mg/kg BW

DISCUSSION

This study demonstrated that cigarette smoke exposure impaired sperm quality and liver histopathology in rats, whereas administration of black garlic extract improved these parameters. Cigarette smoke significantly reduced sperm concentration, viability, and motility, while treatment with black garlic extract partially restored these parameters, particularly at a dose of 500 mg/kg body weight. Histopathological findings also showed reduced cellular degeneration and necrosis in the treatment groups. However, serum ALT and AST levels did not differ significantly among groups.

Cigarette smoke contains numerous toxic compounds that generate ROS, leading to oxidative stress and cellular damage.[3-5] Excessive ROS can damage lipids, proteins, and DNA, impairing spermatogenesis and sperm cell integrity. Lipid peroxidation of the sperm plasma membrane, which is rich in polyunsaturated fatty acids, contributes to decreased motility and viability. In addition, oxidative stress induces DNA fragmentation and apoptosis in spermatozoa, ultimately reducing fertility potential.[6,8]

The improvement in sperm quality observed in this study is likely attributable to the antioxidant properties of black garlic. Black garlic contains bioactive compounds such as phenolics, flavonoids, and SAC, which act as potent free radical scavengers. These compounds enhance endogenous antioxidant defenses and reduce oxidative damage. SAC has been shown to neutralize ROS and preserve mitochondrial function, which is essential for ATP production and sperm motility.[11,12,16-21]

Flavonoids and polyphenols further contribute to antioxidant activity by donating electrons to neutralize free radicals and inhibiting lipid peroxidation. Additionally, flavonoids can chelate transition metals involved in ROS generation, thereby reducing oxidative damage. Polyphenols formed during garlic fermentation have also been reported to preserve chromatin integrity and reduce DNA fragmentation in spermatozoa.[19,22-24]

In addition to reproductive protection, black garlic extract demonstrated hepatoprotective effects. Cigarette smoke exposure induced hepatocellular damage characterized by parenchymatous degeneration, hydropic degeneration, and necrosis. These changes are consistent with oxidative stress–mediated liver injury, in which ROS disrupt cellular redox balance, leading to mitochondrial dysfunction and membrane damage.[9]

Administration of black garlic extract reduced the severity of these histopathological alterations, particularly at a dose of 500 mg/kg body weight. This hepatoprotective effect may be associated with sulfur-containing compounds such as diallyl disulfide and diallyl trisulfide, which enhance glutathione synthesis and support detoxification pathways. In addition, SAC and polyphenols may stabilize hepatocyte membranes and reduce inflammatory responses, thereby protecting liver cells from oxidative damage.[18,19,21]

Interestingly, despite improvements in liver histopathology, serum ALT and AST levels did not show significant changes. This finding suggests that structural recovery of liver tissue may precede detectable changes in circulating enzyme levels.[25-27] Previous studies have reported similar

findings, indicating that biochemical markers and histopathological changes may reflect different stages of tissue injury and recovery.[28]

Another important finding of this study is that the highest dose of black garlic extract (1000 mg/kg body weight) did not produce additional beneficial effects and was associated with the presence of necrotic cells in liver tissue. This observation suggests that excessive doses of antioxidant compounds may not always provide greater protective effects and could potentially induce cellular stress. Therefore, dose optimization is an important consideration in the therapeutic application of natural antioxidant compounds.

Several limitations should be acknowledged. The extraction yield and phytochemical composition of black garlic extract were not analyzed, limiting reproducibility and identification of active compounds. In addition, oxidative stress biomarkers and reproductive hormones were not measured, restricting mechanistic interpretation. Future studies should incorporate phytochemical profiling, oxidative stress markers, and hormonal analysis to better elucidate the underlying mechanisms. Overall, these findings support the potential role of black garlic extract as a natural antioxidant capable of mitigating cigarette smoke–induced reproductive and hepatic damage, with the most effective dose observed at 500 mg/kg body weight.

CONCLUSIONS AND RECOMMENDATION

This study demonstrates that cigarette smoke exposure impairs sperm quality and induces structural liver damage. Administration of black garlic extract attenuated these effects, with the most consistent improvement observed at a dose of 500 mg/kg body weight. However, no significant changes were detected in serum ALT and AST levels. These findings suggest that black garlic extract exerts protective effects against cigarette smoke–induced oxidative damage, likely through its antioxidant properties. Further studies are needed to elucidate the underlying molecular mechanisms and identify the bioactive compounds responsible for these effects. Future research should incorporate oxidative stress biomarkers, reproductive hormonal parameters, and standardized phytochemical analysis to strengthen the evidence supporting its potential development as a functional health supplement.

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