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Protective effects of *Cynanchum auriculatum* Royle ex Wight on testicular reproductive decline in naturally aging male rats

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Abstract: **Background**: The aging process leads to organ degeneration, including testicular aging, which not only impairs fertility but also increases the risk of reproductive system diseases, significantly reducing the quality of life in elderly individuals. Cynanchum auriculatum (*C. auriculatum*) Royle ex Wight exhibits anti-aging, antioxidant and anti-inflammatory properties; however, its effects on testicular reproductive function decline in naturally aging male rats remain underexplored. **Objectives**: This research explored the beneficial effects and underlying mechanisms of *C. auriculatum* on testicular reproductive function in aging male rats. **Methods**: We divided male SD rats into four groups: a middle-aged control group, an aging model group and groups receiving low and high doses of *C. auriculatum*. We collected testicular tissues to assess morphological alterations, DNA integrity and apoptotic activity. **Results**: Relative to the middle-aged control group, the aging model group exhibited marked reductions in both testicular weight and testicular index. Conversely, the *C. auriculatum*-treated groups demonstrated improvements in these indices along with enhanced seminiferous tubule structure. Furthermore, *C. auriculatum* administration led to a decrease in DNA damage and apoptosis, significantly suppressing phosphorylated histone H2AX (γ-H2AX), BCL2-associated X protein (Bax) and phosphorylated p53 (p-P53) protein expression while elevating B-cell lymphoma 2 (Bcl2) protein expression. **Conclusion**: These findings suggest that *C. auriculatum* effectively delays reproductive function decline in the testicular tissues of naturally aging rats, likely by inhibiting DNA damage, modulating the P53 signaling pathway, and reducing reproductive cell apoptosis.

Keywords: Aging; Apoptosis; *C. auriculatum*; DNA damage; Testis

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INTRODUCTION

The rising trend of delayed marriage and childbearing has led to increasing reproductive needs among older couples, particularly in high-income countries and regions, where delayed paternal age is more pronounced (Zhang *et al.*, 2023). Advanced paternal age is associated with age-related declines in testicular function, negatively impacting sperm parameters, reproductive success and offspring health (Liu *et al.*, 2023). Oxidative stress, DNA damage, decreased DNA repair capacity and apoptosis are key contributors to the decline in male reproductive function (Ma *et al.*, 2024). As aging progresses, oxidative stress and DNA damage in the testes increase, causing structural damage to cell membranes and reducing the number of germ cells (GC) within testicular tissue, ultimately impairing normal physiological functions. If DNA damage remains unrepaired, it can result in genetic mutations or chromosomal rearrangements, affecting sperm production and quality (Dong *et al.*, 2022). Strengthening prevention on DNA damage-induced testicular functional decline is therefore essential. Known commonly as Baishouwu, the root of *Cynanchum auriculatum* (*C. auriculatum*) Royle ex Wight belongs to the Asclepiadaceae family and is prevalent in various Asian countries (Wang *et al.*, 2021). The medicinal value of *C. auriculatum* roots is well-documented in historical Traditional Chinese Medicine (TCM) texts, where it is commonly used for tonifying the body and delaying aging (Wang *et al.*, 2017). Studies have shown that *C. auriculatum* exhibits anti-tumor, immune-regulatory, antioxidant, hepatoprotective and gastroprotective activities (Wu *et al.*, 2025). However, the role of *C. auriculatum* in male reproduction remains unclear.

In this study, *C. auriculatum* was found to significantly protect against testicular functional decline in naturally aging male rats. The results showed that *C. auriculatum* increased testicular weight and index, improved seminiferous tubule structural integrity and inhibited the expression of proteins related to DNA damage and apoptosis. These findings suggest that *C. auriculatum* may protect testicular reproductive function by modulating the p53 pathway, offering new insights into delaying reproductive aging.

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MATERIALS AND METHODS

Establishment of animal model

The SD rats were acquired from the Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China, Batch No. 210726211101754222). At Jiangsu Medical College, the rats were kept in a pathogen-free environment with a 12-hour light/dark cycle, 25 °C temperature and unrestricted access to food and water. The rats were divided into four groups at random. Until they were 12 months old, the Control group was given unlimited food. The Model group was fed ad libitum until 24 months of age. The *C. auriculatum*-H and *C. auriculatum*-L groups received *C. auriculatum* (30 g/kg and 10 g/kg, respectively) from 8 months of age until 24 months. Treatment schedules are shown in Fig. 1A.

Hematoxylin-Eosin (H&E) staining

Testicular tissues were excised and weighed. A portion of the tissues was fixed in 4% paraformaldehyde, followed by routine dehydration, transparency and paraffin embedding. Tissue sections (3 μm thick) were stained with H&E to observe morphological changes in testicular tissues.

Immunohistochemistry (IHC)

The testicular tissues were embedded in paraffin, sectioned at a thickness of 3 μm and then fixed in 4% paraformaldehyde. In accordance with the guidelines provided by the IHC kit (Elabscience, E-IR-R221), the sections were deparaffinized and then antigen retrieval was performed. A secondary antibody was applied to the sections after primary antibody incubation and hematoxylin was used as a counterstain. Before coverslipping, we used alcohol to dehydrate the material and an eco-friendly clarifying agent to make it transparent. The sections were subjected to an optical microscope examination after being air-dried and mounted with neutral gum.

TUNEL staining

The TUNEL assay kit (Beyotime, C1098) was used to identify GC apoptosis in accordance with the manufacturer's instructions. Before DAB staining, sections were incubated with 3% hydrogen peroxide for 20 minutes to remove endogenous peroxidase activity. Coverslipping and microscopy were performed after hematoxylin counterstaining. Cells with brownish-yellow nuclei were identified as apoptotic cells.

Western blot (WB)

Total protein was extracted from testicular tissue. Cell lysate (Biyuntian Biotechnology, P0013) was then used to lyse the cells. Following this, the cells were agitated on ice for 30 minutes and then centrifuged at 12,000 × g for 10 minutes at 4 °C. The Biyuntian Biotechnology P0010 BCA protein assay kit (Beyotime, P0010) was used to quantify the total protein concentration. Various antibodies were utilized, including one against γ-H2AX (GB111841), another against p53 (CST, #9284), still another against Bax (CST, #2772), one against Bcl2 (Abcam, ab196495) and one against tubulin (Proteintech, 66031-1-lg).

Statistical analysis

Data were presented as mean ± SEM and statistical significance was evaluated using one-way ANOVA followed by Tukey's multiple comparison test with GraphPad Prism 10. A p-value of less than 0.05 was considered statistically significant. Statistical notations included $*p < 0.05$ compared to the control group, $\#\#\#p < 0.001$, $\#p < 0.05$ versus the model group. Histology and immunohistochemistry were performed and analyzed blindly before sample identities were decoded.

RESULTS

C. auriculatum increases testicular weight, testicular index and improves seminiferous tubule morphology

To investigate the role of *C. auriculatum* in testicular aging, SD rats were fed low and high doses of *C. auriculatum* for 16 weeks (Fig. 1A). Measurements of body weight (BW) and testis weight were recorded and the ratio of testis weight to BW was calculated. Relative to the control group, the model group displayed a significant reduction in both testicular weight and testicular index. Conversely, the *C. auriculatum*-L and *C. auriculatum*-H groups exhibited increases in these metrics compared to the model group, with the *C. auriculatum*-H group showing greater improvements (Fig. 1B, C).

H&E staining of testicular sections revealed an intact seminiferous tubule basement membrane, abundant GC and closely packed cells within the lumen in the control group. In the model group, the basement membrane was thinner, GC were disorganized, cell alignment was disrupted and the number of mature sperm within the lumen was reduced. In the *C. auriculatum*-L and *C. auriculatum*-H groups, the morphology and number of GC in each layer of the seminiferous tubules were similar to those in the control group and cells were well organized (Fig. 1D). These findings indicate that *C. auriculatum* improves the reproductive function of testicular tissue in rats.

C. auriculatum alleviates aging-induced DNA damage in testicular tissues

To explore whether *C. auriculatum* alleviated aging-induced DNA damage *in vivo*. WB and IHC analyses were performed to assess γ-H2AX protein expression, a biomarker for DNA damage, in testicular tissues. Elevated levels of γ-H2AX in the model group indicated significant DNA damage, whereas treatment with *C. auriculatum* at both low and high doses markedly reduced γ-H2AX expression (Fig. 2A-D). These results suggest that *C. auriculatum* mitigates age-induced DNA damage in the testes by modulating γ-H2AX protein expression.

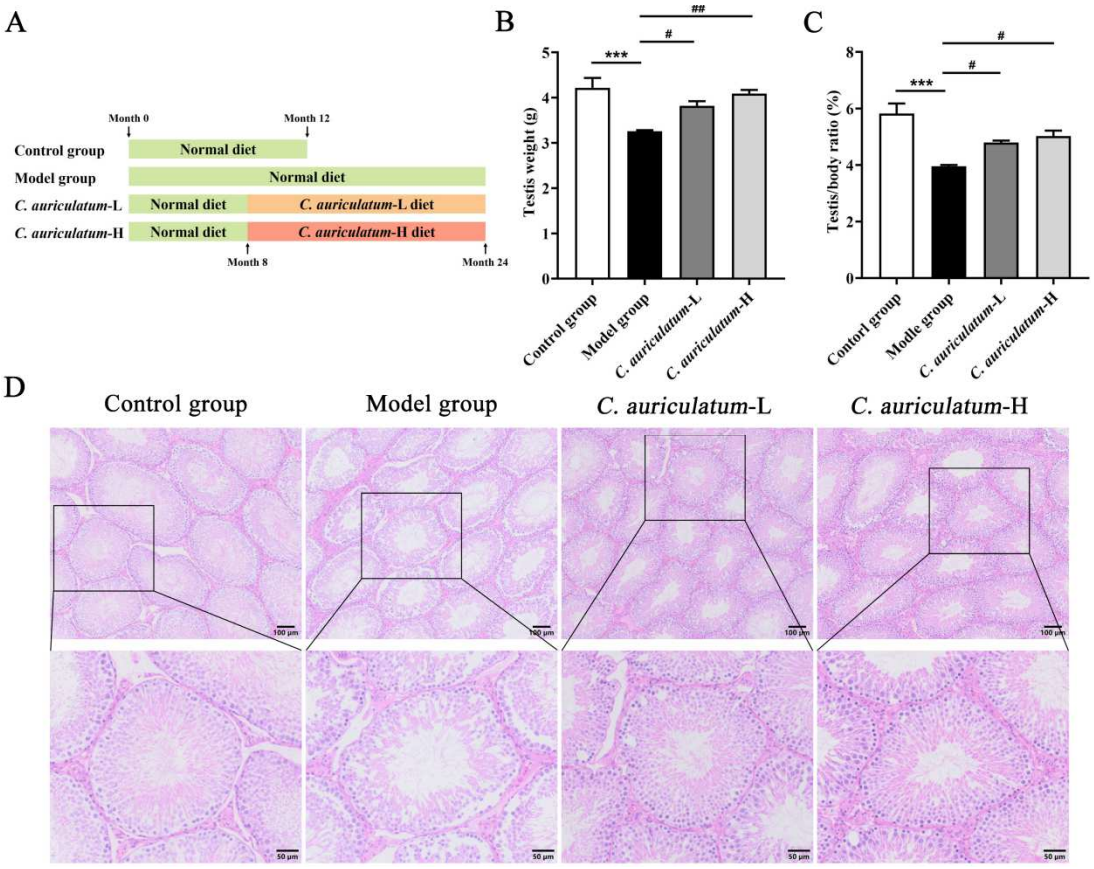

Fig. 1: Effects of *C. auriculatum* on testicular weight, testicular index, and seminiferous tubule morphology.
(A) Flow chart of animal experiments. (B) Testicular weight (n=5). (C) Testicular index (n=5). (D) HE staining of rat testis (n=5).

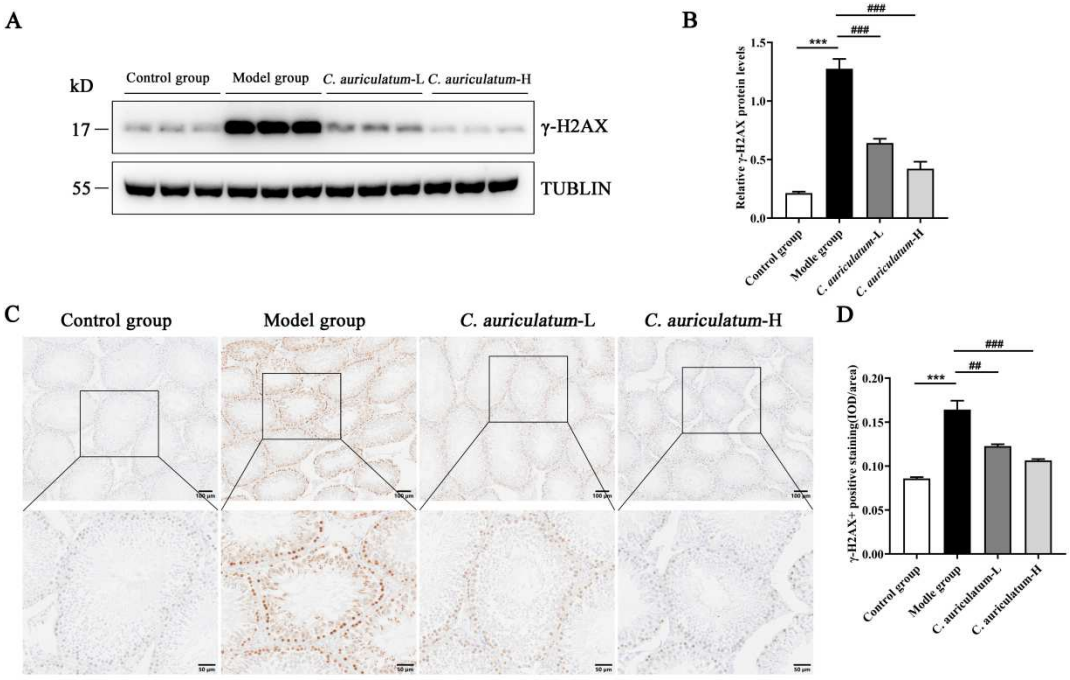

Fig. 2: *C. auriculatum* alleviates aging-induced DNA damage in testicular tissues.
(A, B) WB analysis and quantification of γ-H2AX expression in rat testes (n=3). (C, D) IHC staining and quantification of γ-H2AX expression in rat testes (n = 3).

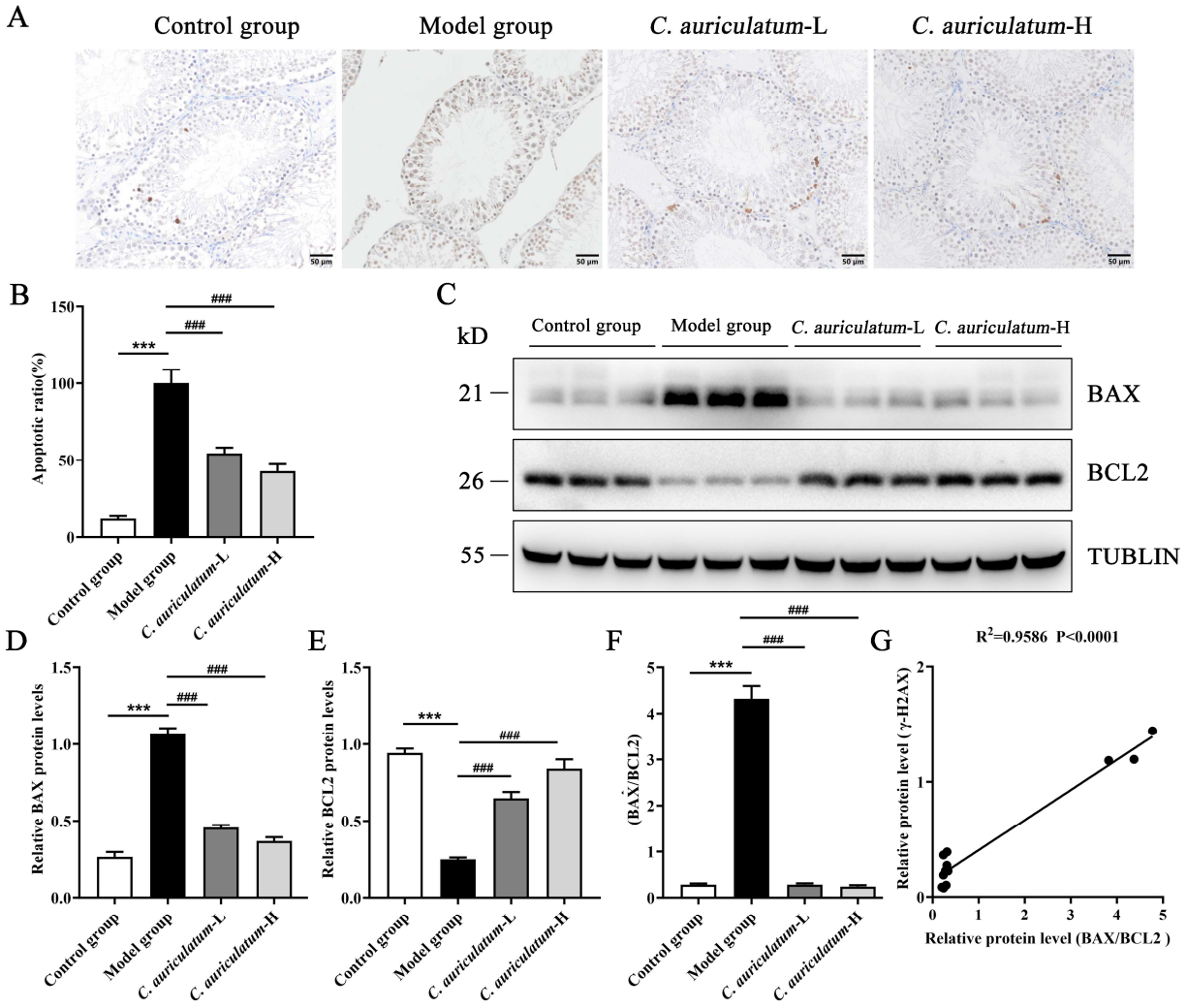

Fig. 3: *C. auriculatum* inhibits aging-induced GC apoptosis in testicular tissues.
(A, B) IHC staining and quantification of apoptotic cells in rat testes (n=3). (C-E) WB analysis and quantification of Bax and Bcl2 expression in rat testes (n=3). (F) Bax/Bcl2 ratio (n=3). (G) Correlation between the Bax/Bcl2 ratio and γ-H2AX protein levels (n=3).

C. auriculatum inhibits aging-induced apoptosis of GC in testicular tissues

Previous studies have shown that apoptosis often occurs in response to DNA damage (Zhang *et al.*, 2024). In this study, TUNEL staining was used to evaluate GC apoptosis in the seminiferous tubules of testicular tissues across different groups. Apoptotic GC were identified by the brown-yellow staining of their nuclei. In terms of apoptosis, the model group showed an increase in apoptotic GC, suggesting an age-related decline in reproductive capacity. In contrast, both *C. auriculatum*-treated groups demonstrated a significant decrease in apoptotic GC, with a more pronounced effect observed in a dose-dependent manner (Fig. 3A, B).

The study further analyzed the expression of apoptotic regulatory proteins Bax and Bcl2. The model group exhibited increased Bax and decreased Bcl2 levels, resulting in a higher Bax/Bcl2 ratio. Treatment with *C. auriculatum* reversed these effects, diminishing the Bax/Bcl2 ratio in both low- and high-dose groups (Fig. 3C-F). The correlation between γ-H2AX levels and the Bax/Bcl2 ratio was also analyzed in the same animals. A significant positive correlation was observed, suggesting that DNA damage is directly associated with apoptosis (Fig. 3G). These results indicate that *C. auriculatum* may inhibit GC apoptosis by regulating Bax and Bcl2 expression.

C. auriculatum inhibits activation of the p53 pathway

Research has shown that p53 is a critical protein linking DNA damage to cell apoptosis. Upon DNA damage, p53 activation can promote Bax and suppress Bcl2 expression, triggering apoptosis (Zhang *et al.*, 2024). This study evaluated p-P53 expression in testicular tissues; the model group showed a significant upregulation, indicative of heightened p53 pathway activity during aging. However, *C. auriculatum* treatment substantially lowered p-P53 expression levels (Fig. 4A, B). These findings suggest that *C. auriculatum* may inhibit testicular cell apoptosis by suppressing p53 signaling pathway activation.

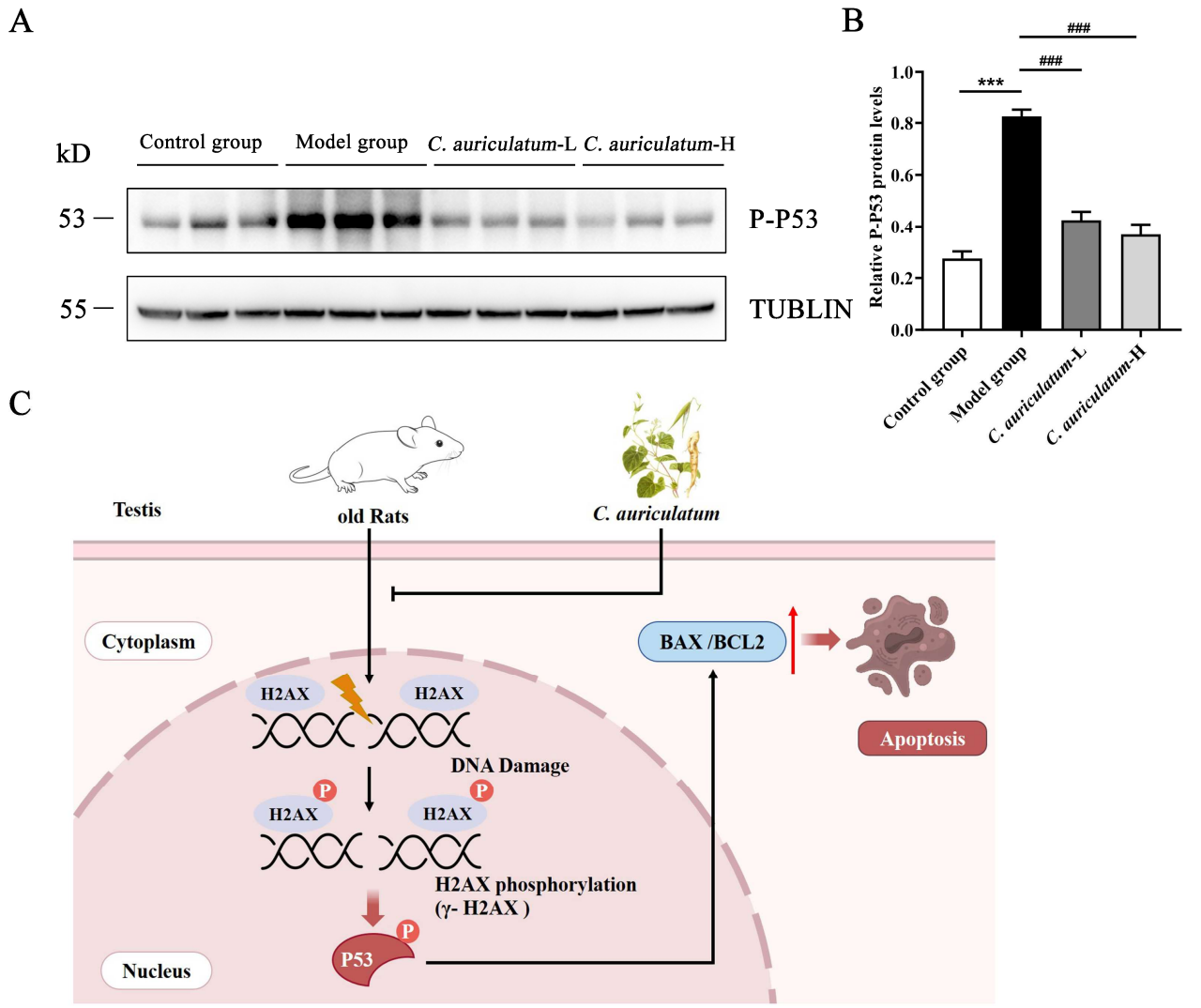

Fig. 4: *C. auriculatum* inhibits activation of the p53 pathway.
(A, B) WB analysis and quantification of p-P53 expression in rat testes (n=3). (C) Proposed working model: *C. auriculatum* regulates testicular cell apoptosis via the p53/Bax/ Bcl2 axis in rat testes.

DISCUSSION

Aging is a degenerative process characterized by the gradual deterioration of various organ systems (Li *et al.*, 2024). In males, aging leads to a progressive decline in testicular function. This decline is accompanied by reductions in testicular weight and size, lower sex hormone levels and abnormalities in testicular morphology (Kalkanli *et al.*, 2021). Therefore, identifying measures and drugs to prevent reproductive decline associated with aging is critical.

Many studies have reported that *Polygonum multiflorum* has anti-aging properties. However, its role in testicular aging remains unclear. This study demonstrated that administration of *C. auriculatum* significantly increased testicular mass and testicular index in naturally aging rats. Additionally, the number and layers of spermatogenic cells notably recovered. Improvements were observed in various stages of spermatogenesis. These findings indicate that *C. auriculatum* protects against testicular reproductive decline in naturally aging rats.

Sperm DNA integrity is a critical indicator of male fertility. DNA damage intensifies with advancing age (Kulashreshtha *et al.*, 2016). DNA double-strand breaks represent the most severe form of DNA damage in eukaryotic cells (Oizumi *et al.*, 2024). Phosphorylated γ-H2AX, closely associated with DNA double-strand breaks, is a biomarker for DNA damage, apoptosis and aging (Collins *et al.*, 2020). WB and IHC results showed that γ-H2AX expression was significantly elevated in the testicular tissue of naturally aging rats. *C. auriculatum* administration notably downregulated its expression. This results suggest that *C. auriculatum* reduces DNA damage in reproductive cells. Thus, it delays reproductive function decline in aging rats.

DNA damage frequently initiates apoptosis (Wang, 2001). Apoptosis is a tightly regulated process of programmed cell death. It is closely linked to the decline in organ function during aging and the progression of aging-related diseases (Symonds *et al.*, 2009). Members of the Bcl2 family, including Bcl2 and Bax, play critical roles in regulating apoptosis (Samia *et al.*, 2024). The anti-apoptotic protein Bcl2 forms homo- or heterodimers with the pro-apoptotic protein Bax. This interaction contributes to cell survival (Samia *et al.*, 2024; Yan *et al.*, 2021). The Bax/Bcl2 ratio critically determines apoptosis in normal and malignant cells (Wang *et al.*, 2024; Kiang *et al.*, 2022). This study found that *C. auriculatum* significantly reduced apoptotic cell numbers in the testicular tissue of aging rats. In naturally aging rats, Bcl2 expression was markedly reduced, while Bax expression was significantly elevated. Conversely, *C. auriculatum* treatment significantly increased Bcl2 expression and decreased Bax expression. These findings suggest that *C. auriculatum* effectively reduces apoptosis in aging testicular tissue.

Research has shown that DNA double-strand breaks activate downstream p53 signaling through γ-H2AX recruitment (Carlsson *et al.*, 2022; Ali *et al.*, 2024). Activated p53 initiates and regulates the apoptosis pathway (Zhang *et al.*, 2024; Li *et al.*, 2022). It inhibits Bcl2 expression, upregulates Bax expression and promotes apoptosis (Zhang *et al.*, 2019). This study further examined changes in p53 expression. The results revealed a significant increase in p53 protein expression in the testicular tissue of the aging model group. However, all doses of *C. auriculatum* reduced p53 expression. These findings suggest that *C. auriculatum* inhibits activation of the p53 signaling pathway. Consequently, reproductive cell apoptosis is reduced.

CONCLUSION

This study provides the first evidence that *C. auriculatum* regulates testicular cell apoptosis by modulating the P53/Bax/Bcl2 axis (Fig. 4C). Specifically, *C. auriculatum* inhibits DNA damage in reproductive cells, promotes p53 phosphorylation, reduces Bax expression and increases Bcl2 expression. thus protecting against reproductive function decline in the testicular tissues of naturally aged rats. Further work is required to clarify additional mechanisms involved in the regulation of testicular reproductive function by *C. auriculatum* in naturally aging rats.

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Author's contributions
Xuan Shen: Designed the project; Performed the experiments; Writing – original draft; Funding acquisition. Xuan Liu: Performed the experiments; Analyzed the data; Software. Wenying Zhang: Performed the experiments; Analyzed the data. Qin Wu: Writing – review & editing; Supervision; Funding acquisition; Resources. Yuping Chen: Writing – review& editing; Funding acquisition; Data curation; Project administration. All authors have read and agreed to the published version of the manuscript.

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Data availability statement
All data generated or analyzed during this study are included in this article.

Ethical approval
Jiangsu Medical College's Institutional Animal Care & Use Committee gave their stamp of approval to all animal experiments (Ethical No.XMLL-2022-027).

Conflict of interest
The authors declare no competing interests.

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