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RESEARCH ARTICLE

The Effect and Mechanism of Cinnamaldehyde on Testosterone Secretion in Murine Model

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ABSTRACT

Cinnamon (*Cinnamomum cassia* (*L.*) *D. Don*) is a commonly used drug in traditional Chinese medicine; it is especially useful for improving the reproductive performance in males and females. Cinnamaldehyde is the main component of cinnamon, which has bactericidal and disinfectant properties, promotes gastrointestinal motility and regulates testosterone secretion in the male. The present study aimed to investigate the mechanisms by which cinnamaldehyde regulates testosterone secretion in male animals. For this purpose, four-month-old male mice (n=25) were randomly divided into five groups (n=5 each) and treated with PBS, DMSO and three doses of cinnamaldehyde (25, 50 and 75mg/kg) by gavage for 2 weeks. Histological (IHC), ELISA and molecular analyses (WB, RT-qPCR) were used to assess effects of cinnamaldehyde on murine liver, serum testosterone levels, and testicular enzymes associated with testosterone production. The mechanisms by which cinnamaldehyde affects testosterone secretion in testis Leydig cells were explored using ELISA, flow cytometry, WB, fluorescent quantitative PCR, and dual luciferase reporter systems. The results showed that cinnamaldehyde elevated murine body weight, serum testosterone, testicular interstitial cell density, and steroidogenic enzyme expression (StAR, CYP11A1, 3β-HSD) without altering hepatic tissue and testicular indices. Cinnamaldehyde treatment at 5×10^{-5}M increased the expression of steroid hormone synthase in Leydig cells, increased the proportion of cells in the S phase, significantly increased the promoter activity of the steroid hormone synthesis nuclear receptor steroidogenic factor 1 (SF-1), and up-regulated the expression of the nuclear receptor SF-1. The present study showed that cinnamaldehyde can upregulate the expression of steroid hormone synthase by increasing the activity of the steroid hormone synthesis nuclear receptor promoter, which in turn increases the synthesis and secretion of testosterone from testicular interstitial cells.

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INTRODUCTION

Traditional Chinese medicine is an important part of the excellent traditional Chinese culture, a crystallization of Chinese wisdom since ages. Traditional Chinese medicine has distinct national characteristics, a unique theoretical system, distinctive diagnostic and treatment methods, and effective clinical efficacy. The role of herbal extracts in disease treatment has gradually received increasing attention. Research shows that herbal extracts play a critical role in enhancing immunity, treating cancer, inhibiting bacteria, and regulating reproductive hormones secretion in different animals (Mirmosayyeb *et al.*, 2017; Cao *et al.*, 2019).

Cinnamon is a globally used spice; in China, it is mainly grown in southwestern and southeastern regions (Doyle and Stephens, 2019). In traditional Chinese medicine, it is mostly used to alleviate pain, reduce inflammation, and balance metabolic heat (Liu *et al.*, 2024a; Luo *et al.*, 2024). Previous studies have highlighted its broad therapeutic potential, including antimicrobial, antioxidant, antitumor, and cardiometabolic

benefits (Chou *et al.*, 2013; Mirmosayyeb *et al.*, 2017). These properties may arise from its modulation activity of steroidogenic enzymes such as steroidogenic acute regulatory protein (StAR), cytochrome p450 family 11 subfamily a member 1 (Cyp11a1), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome p450 family 11 subfamily a member 1 (Cyp17a1), and 17β-hydroxysteroid dehydrogenase (17β-HSD). The expression and activities of these enzymes are regulated by diverse physiological factors (Wang *et al.*, 2023).

In the animal husbandry program, cinnamon is used as an additive to the feed in order to improve the level of testosterone and the reproductive function of male animals (Ranasinghe *et al.*, 2013). Testosterone is the main male sex hormone, which is a crucial hormone responsible for the development and maintenance of the male reproductive system, the appearance of secondary sexual characteristics, the development of accessory glands, and for the process of spermatogenesis. Testosterone is secreted by the interstitial cells (Leydig cells) of the seminiferous tubules of the testis; it activates a number of signaling pathways and is a target of multiple enzymes. It has been shown from several studies that cinnamaldehyde (CMA), the primary component of cinnamon, is able to regulate hormones production and release. Cinnamaldehyde is known to regulate the synthesis and secretion of insulin, progesterone, and testosterone (Iwaoka *et al.*, 2010; Safaei *et al.*, 2021; Gopalakrishnan *et al.*, 2023). Additionally, numerous studies have indicated that cinnamon exhibits a range of beneficial properties, including antibacterial, antiviral, antifungal, antioxidant, antitumor, antihypertensive, lipid-lowering, and hypoglycemic effects, as well as protective effects on the stomach and immune modulation (Ka *et al.*, 2003; Chou *et al.*, 2013; Mirmosayyeb *et al.*, 2017).

Leydig cells in the seminiferous tubules of the testis use cholesterol as a raw material to synthesize testosterone under the catalytic effect of steroidogenic enzymes. The enzymes involved in this process include StAR, Cyp11a1, 3β-HSD, 17β-HSD, and Cyp17a1 (Wang *et al.*, 2023). Various factors regulate the expression and activities of these steroidogenic enzymes. The hypothalamic-pituitary-testicular axis is the classical pathway regulating testosterone synthesis and secretion in interstitial cells of the testis. Testosterone synthesis primarily occurs in the mitochondrial membrane of the testicular interstitial cells, making mitochondrial membrane remodeling essential for testosterone synthesis (Pereira *et al.*, 2024). Upon LH-cAMP signaling stimulation, mitochondria in testicular interstitial cells undergo structural changes, enhancing StAR activity, facilitating cholesterol transport, and increasing testosterone production. Certain steroidogenic nuclear receptors, such as steroidogenic factor 1 (SF-1) and nuclear receptor subfamily 4 group A member 1 (Nur-77), regulate testosterone synthesis by modulating the activity of steroidogenic enzyme promoters and ultimately influencing enzyme expression (Xiao *et al.*, 2021). Nuclear receptors such as small heterodimer partner (SHP) and orphan nuclear receptor DAX1 inhibit the transcription and expression of steroidogenic enzymes indirectly by binding to steroidogenic nuclear receptors, thereby inhibiting testosterone synthesis. In the testicular interstitial cells of mature male animals, SHP inhibits the activity of SF-1, Nur-77, and LRH1 through its DNA-binding domain, suppressing the expression of various steroidogenic enzymes and inhibiting testosterone production (Xiao *et al.*, 2021; Liu *et al.*, 2024b; Zhang *et al.*, 2024).

Keeping in view the important role of cinnamon in regulating male reproductive function, it was hypothesized that cinnamaldehyde (CMA), the main component of cinnamon, may play an important role in regulating testosterone synthesis in the mammalian testis. Therefore, this study explores the effects of CMA on the signaling pathways related to steroid hormone synthesis by regulating testosterone secretion in Leydig cells, revealing the mechanism by which CMA regulates reproductive hormone synthesis and secretion. This research can provide an experimental basis for further expanding the application fields of cinnamon, increasing its economic value, and improving the reproductive performance of livestock.

MATERIALS AND METHODS

Animals: The present study was conducted during the period from June 2022 to August 2024. A total of 25, four-month-old male Kunming white strain mice, with mean±SD body weight of 39.01±0.86g, were obtained from Wushi Laboratory Animal Center (Fujian, China) for this study. These mice were maintained at 25℃ with a 12-hour light/dark cycle, and provided with ad libitum access to standard feed and clean water. All animal procedures received prior approval from the Institutional Animal Care and Use Committee (IACUC) of Fujian Agriculture and Forestry University, Fuzhou, Fujian Province, China. The number of laboratory animal use license is SYXK 2019-0004.

Cinnamaldehyde treatment: The experimental mice were randomly divided into five treatment groups (n=5 each), as follows: Rats of group-1 were treated with PBS (100mL) and those of Group-2 were given 100 mL of 2% DMSO in PBS (Lot. 20140415; Sinopharm Chemical Reagent). Rats of Groups 3, 4 and 5 were given 100 mL of CMA at 25, 50 and 75mg/kg BW, respectively (Lot. STBJ1063; Sigma-Aldrich Biotech) dissolved in 2% DMSO. Because the mean body weight of experimental mice was about 40g, the mass of CMA per mouse in the 25mg/kg group was 1mg. So, in 25mg/kg group, 99mL of 2% DMSO was used to dissolve 1mg CMA. By analogy, in the 50mg/kg group, 98mL of 2% DMSO was used to dissolve 2mg CMA. In the 75mg/kg group, 97mL of 2% DMSO was used to dissolve 3mg CMA. These treatments were given to rats of the respective groups by gavage for 2 weeks. Because CMA was dissolved in DMSO, the 2% DMSO group was used as the solvent control. After 2 weeks, mice were euthanized by cervical spondylolisthesis and blood serum, testis and hepatic tissue samples (≈1cm^3) were collected.

The murine Leydig tumor cell line (MLTC-1) was purchased from Cell Institute of Shanghai (Shanghai, China). This cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Lot. 8120391; Thermo Fisher Scientific) supplemented with 10% fetal

bovine serum (FBS; Lot. I91123, Transgen Biotech.) and 1% penicillin/streptomycin (Lot. SH40003.01, Thermo Fisher Scientific).

In order to screen the CMA concentration and treat time, MLTC-1 were seeded in 24-well plates (5×10^4 cells/well). After 24h, the medium was replaced with RPMI-1640 containing six concentrations of CMA (0×10^{-5}, 5×10^{-5}, 7.5×10^{-5}, 10×10^{-5}, 20×10^{-5} and 40×10^{-5}M). After 24h and 48h, cell numbers were counted and the culture supernatant was collected.

For detecting the effects of CMA treatment on the expression of steroidgenesis synthase, steroidgenesis related nuclear receptor and cyclins, cells were seeded as shown above. After 24h, the medium was replaced with RPMI-1640 medium containing 0×10^{-5}M and 5×10^{-5}M CMA. After 24h of continued culture, the cells and the culture supernatant were collected.

Testosterone measurement: Blood serum and MLTC-1 cell culture supernatant testosterone (T) levels were measured using a Beifang Biotech ELISA (Lot. 20162400326) kit according to the manufacturer's instructions, with a sensitivity threshold of 0.05ng/mL. Assay precision showed that intra- and inter-assay coefficients of variation were <10% and <15%, respectively.

Hematoxylin Eosin (HE) stain and Immunohistochemistry (IHC): Post-euthanasia, hepatic and testicular specimens were collected and fixed in 4% paraformaldehyde (24h), followed by ethanol gradient dehydration and paraffin embedding. Serial 7μm sections cut with a rotary microtome were mounted on poly-L-lysine-coated slides. Liver tissue sections were subjected to H&E staining: hematoxylin (3 min) → water rinse → acid-alcohol differentiation (1% HCl/EtOH, 5s) → ammonia blueing (0.6% NH4OH) → eosin counterstain (2 min), as described earlier (Xiong *et al.*, 2017). Neutral balsam-mounted slides were imaged using a Motic BA400 digital microscopy system.

Testicular sections (7μm thick) were antigen-retrieved via citrate buffer (0.1M, pH 6.0) with microwave irradiation (750W, 10min×2 cycles). Post-PBS rinsing, endogenous peroxidases were blocked using 0.3% H_2O_2 in methanol (37°C/1h). IHC staining employed a commercial detection system (Maixin KIT-9706) with 3β-HSD primary antibody (Affbiotech DF6639, 5μg/mL), followed by digital imaging analysis (Xiong *et al.*, 2017).

Western blotting: Testicular tissue and MLTC-1 cell lysates were prepared via KeyGen Protein Extraction Kit. Following quantification, proteins underwent 12% SDS-PAGE separation and PVDF membrane transfer (Millipore). The step-wise immunoblotting procedure is described below. 1): Blocking with 10% skim milk/TBST (Tris-buffered saline with 0.05% Tween 20) (1h/RT); 2): Primary antibody incubation (Table 1), β-actin-normalized, 4°C/overnight); 3): HRP-secondary antibody incubation (Zhongshan, 1:5000, 1h/RT) after TBST washes (3×). Chemiluminescent quantification followed established methodologies, with triplicate validation (Wang *et al.*, 2019).

Table 1: Antibodies used in Western-blotting experiment

Antibodies	Company and Cat#	Antibodies concentrations (μg/mL)
StAR	Huabio, ER1917-23	1.0
3β-HSD	Affbiotech, DF6639	1.0
CYP11A1	Huabio, ER1906-98	1.0
CYP17A1	Huabio, ET7107-61	1.0
β-actin	Sungene Bio, LK9001	0.5

RT-qPCR analysis: Total RNA of MLTC-1 was isolated using TRIzol™ (TaKaRa), with purity confirmed by NanoDrop OneC (A260/A280 >1.8, A260/A230 >2.0). Following reverse transcription with PrimeScript™ RT reagents (TaKaRa), quantitative PCR was performed on a Bio-Rad CFX96 system using SYBR Premix Ex Taq™ II (TaKaRa) and TsingKe Biotech primers (Table 2), under standardized conditions: 95°C/30s initial denaturation; 40 cycles of 95°C/5s and 60°C/30s. Gapdh served as an endogenous control for 2^-ΔΔCt analysis of biological triplicates (Xiong *et al.*, 2017; Wang *et al.*, 2019).

Table 2: The primers sequences used for RT-qPCR

Name of primers	Primers' sequences	Gene bank number
GAPDH	F-5'- CCAGTATGACTCCACTCACGG -3' R-5'- TCCACGACATACTCAGCACC -3'	XM_036165840.1
Cyp11a1	F-5'-AGGTCCTTCAATGAGATCCCTT-3' R-5'-TCCCTGTAAATGGGGCCATAC-3'	NM_019779.4
3β-HSD	F-5'-TCCCCATTCAGAGCATGTATAGC-3' R-5'-TTTTTTTGAGGTATTGACAAGTATTTATTG-3'	NM_013821.3
Cyp17a1	F-5'-AGTGCTGAGTACTGGAAAGGG-3' F-5'-TGGGGCCATACTTTTGGAAAC -3'	NM_007809.3
StAR	F-5'-GTGCTGGAAGCTCCTATAGACA -3' R-5'-AGGACAGCTCCTGGTCACTAT-3'	NM_011485.5
SF-1	F-5'-CCCAAGAGTTAGTGCTCCAGT-3' R-5'-CTGGGCGTCCTTTACGAGG-3'	NM_001316687.1
Nur-77	F-5'-TTGAGTTCGGCAAGCCTACC-3' R-5'-GTGTACCCGTCCATGAAGGTG-3'	NM_010444.2
SHP	F-5'-CGATCCTCTTCAACCCAGATG-3' R-5'-AGGGCTCCAAGACTTCACACA-3'	NM_011850.3
Cyclin A1	F-5'-GCCTTCACCATTCATGTGGAT-3' R-5'-TTGCTGCGGGTAAAGAGACAG-3'	Z26580.1
Cyclin B1	F-5'-AAGGTGCCTGTGTGTGAACC-3' R-5'-GTCAGCCCCATCATCTGCG-3'	NM_172301.3
Cyclin D2	F-5'-AAGGTGCCTGTGTGTGAACC-3' R-5'-GTCAGCCCCATCATCTGCG-3'	NM_009829.3

Flow cytometry: Post-harvest MLTC-1 cells (3×10^5/well, 6-well plates) were PBS-washed and processed for cell cycle analysis (Multi science CCS012 kit). FACS quantification used a NovoCyte 1030 system (NovoExpress software, ≥20,000 events/sample), as described previously (Wang *et al.*, 2021). Data were validated by triplicate experiments.

Luciferase reporter assay: As shown above, MLTC-1 were plated in 24-well plates 24h before plasimids transfection. The cells were then divided into three groups and transfected with 1000ng PGL4.10, SF-1 promoter-luc, and Nur-77 promoter-luc for 12h using Turbofect transfection reagent (R0532; Thermo Fisher Scientific). Meanwhile, 200ng PRL-TK was transfected to each group. After transfection, each transfected group was divided into two groups and treated with 0×10^{-5}M and 5×10^{-5}M CMA for 24 h. After treatment, harvested cells and luciferase activity were measured using Dual-Luciferase Assay kit (Promega E1910) according to Wang *et al.* (2023). Firefly/Renilla ratios were used to determine promoter activity comparisons.

Statistical analysis: Dataset comparisons were conducted through one-way ANOVA with Tukey's post hoc verification, supplemented by Fisher's LSD test for multi-group contrasts using SPSS 25. Quantitative results from triplicate experiments are presented as mean±SEM, with a statistical significance threshold was set at $P<0.05$.

RESULTS

Body weight, testis index and serum testosterone concentrations: After treatment of mice through gavage with PBS, DMSO and different doses of cinnamaldehyde for 2 weeks, changes in body weight, testis index, and serum testosterone concentration were measured across the treatment groups. The results demonstrated that, compared to PBS and DMSO, cinnamaldehyde at 25, 50 and 75mg/kg significantly increased body weight and elevated the serum testosterone concentration ($P<0.05$), the differences in body weight and serum testosterone levels among three CMA concentrations were non-significant. Similarly, there was no difference in testis index among rats of five groups (Fig. 1).

Effect of CMA treatment on the liver tissue: The effects of treatment of mice with PBS, DMSO and different doses of cinnamaldehyde on mouse liver tissue were observed using H&E staining. Result showed no abnormalities in the liver tissue of mice in the 25mg/kg cinnamaldehyde group compared to the PBS and DMSO groups (Fig. 2). However, mice subjected to 50 and 75mg/kg cinnamaldehyde treatment exhibited some degree of cell swelling and loosening of the cytoplasm in liver tissue.

Effect of CMA treatment on testicular tissue: The effects of feeding PBS, DMSO and three doses of cinnamaldehyde on mouse testicular tissue were also observed using H&E staining (Fig. 3). Compared to the PBS and DMSO groups, the number of Leydig cells in the testes of cinnamaldehyde-fed mice was increased, with the most pronounced increase was observed in the 25mg/kg treatment group. However, the data could not be analyzed statistically.

Effect of CMA treatment on testosterone synthesis enzymes expression: The expression of testosterone synthesis enzymes in testicular tissue was detected using immunohistochemistry and Western Blotting. Compared to the PBS and DMSO groups, the expression of 3β-HSD enzyme in all cinnamaldehyde-treated groups significantly increased ($P<0.05$; Fig. 4A and Fig. 4B); however, the differences in 3β-HSD expression among three levels of CMA were non-significant (Fig. 4B). Western blot analysis also revealed upregulated expression of key testosterone biosynthesis enzyme StAR in testicular tissue of cinnamaldehyde-treated groups relative to PBS and DMSO controls (Fig. 4C). Moreover, 75mg/kg CMA showed higher StAR expression compared to 25 and 50mg/kg groups ($P<0.05$), the difference in StAR expression between the latter two groups was, however, non-significant. The expression of CYP11A1 was significantly increased only in 75mg/kg CMA group compared to PBS or DMSO group (Fig. 4C).

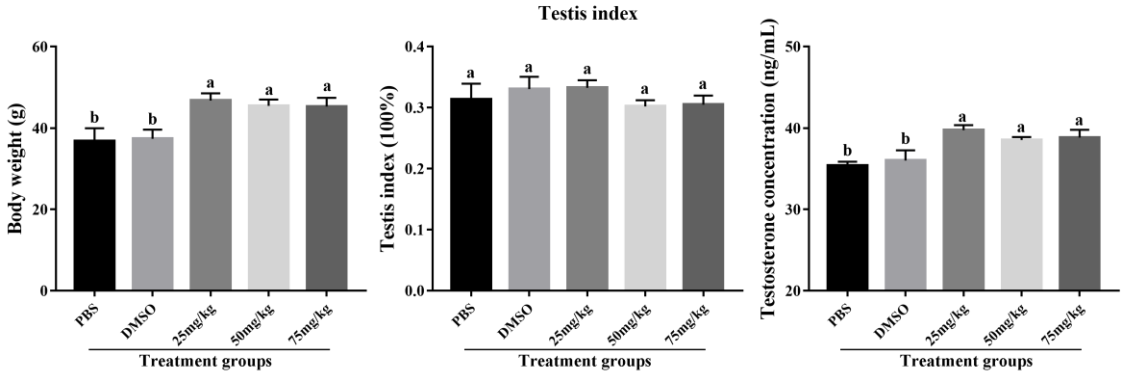

Fig. 1: CMA dietary effects on organ indices and testosterone levels. Triplicate biological replicates (mean±SEM); Different lettering indicates significant intergroup differences for each parameter ($P<0.05$).

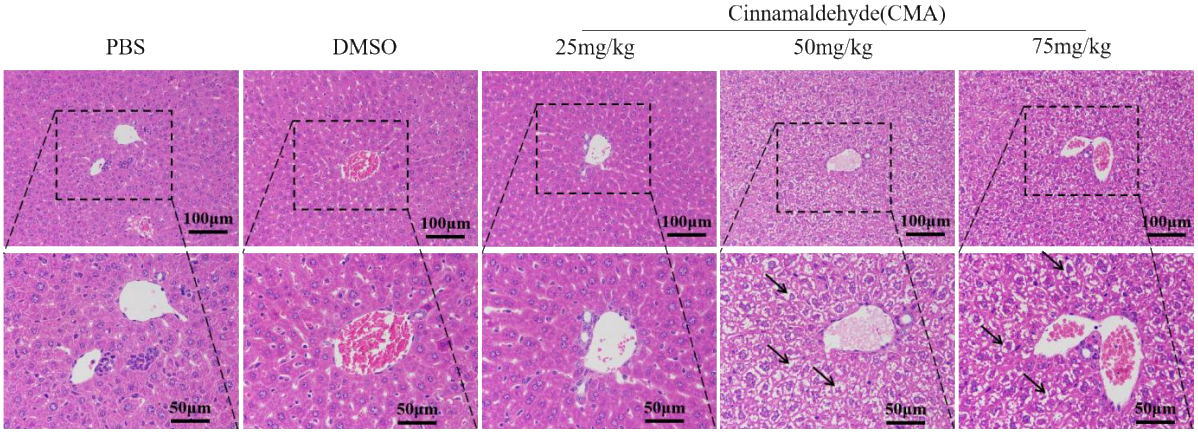

Fig. 2: Effect of feeding CMA on mouse liver tissue. H&E stain microscope images of liver tissue. Representative images of three independent experiments are shown. The changes of liver cells are indicated by arrows.

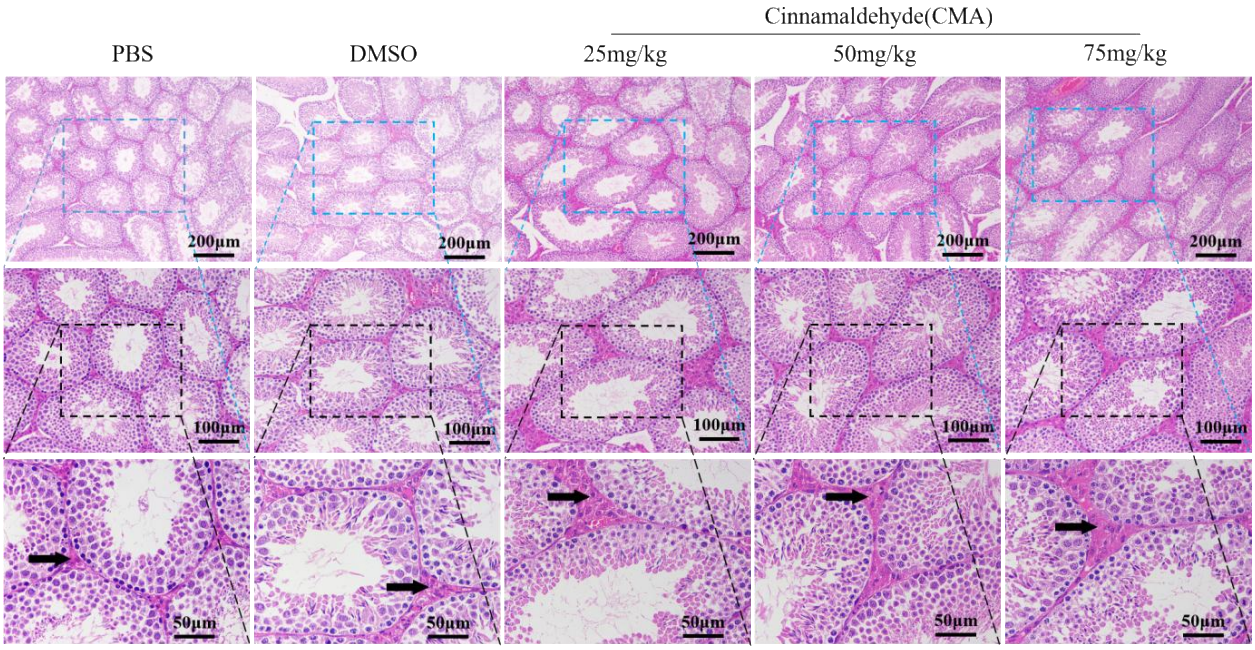

Fig. 3: CMA-induced testicular histomorphological alterations. H&E-stained tissue sections. Representative images of three independent experiments are shown. The Leydig cells are indicated by arrows.

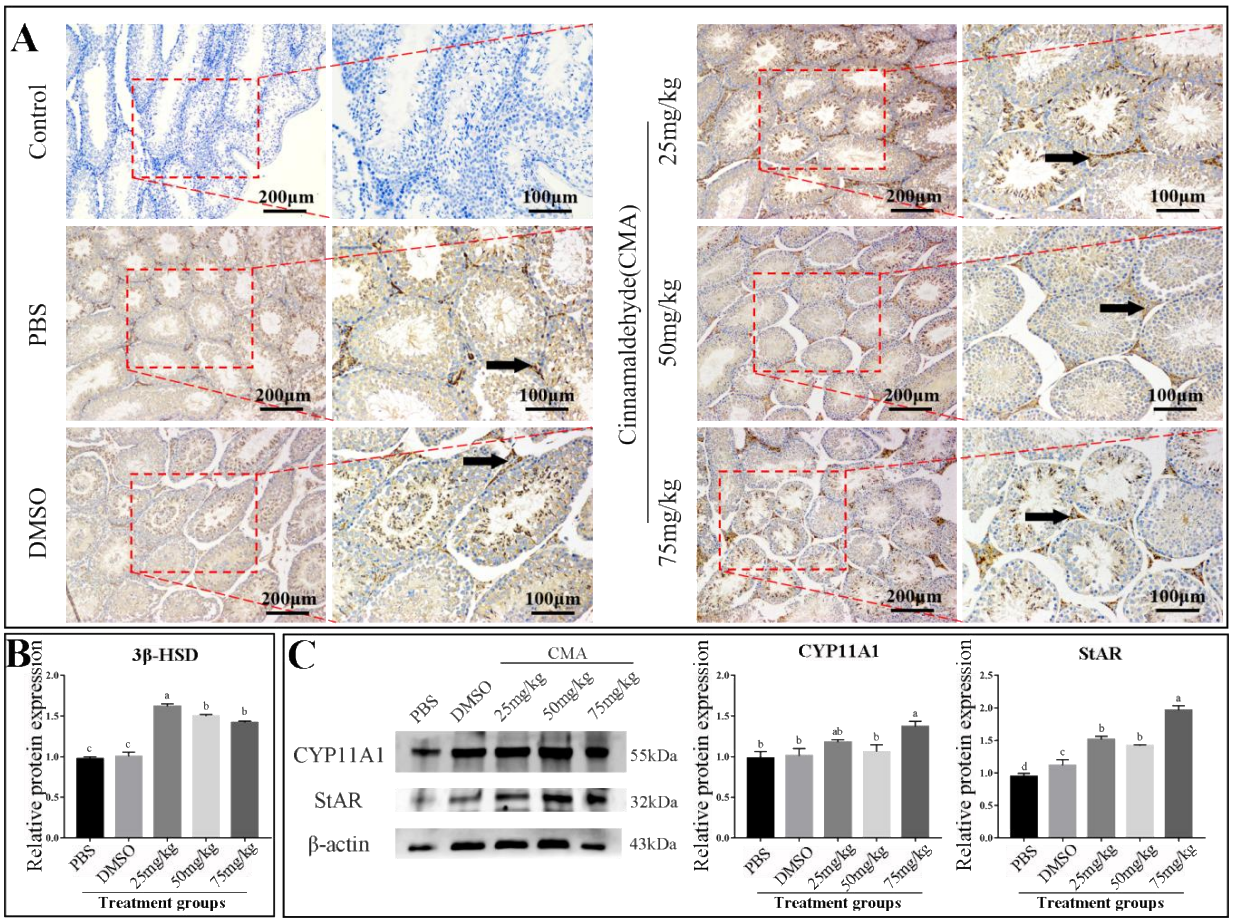

Fig. 4: CMA-mediated testicular steroidogenic regulation. A): IHC analysis of 3β-HSD protein localization. The Leydig cells are indicated by arrows. Representative images of three independent experiments are shown. B): Image J quantification analysis result of IHC. C): WB: CYP11A1 and StAR expression. Quantified data (mean±SEM) with different letters indicate significant differences among groups for each parameter ($P<0.05$).

Effect of CMA treatment on MLTC-1 testosterone secretion: To further study the effects of cinnamaldehyde on testosterone secretion in Leydig cells, MLTC-1 cells were treated with six concentrations of CMA (0, 5×10^{-5}, 7.5×10^{-5}, 10×10^{-5}, 20×10^{-5} and 40×10^{-5}M), and the secretion of testosterone was measured using ELISA. The results showed that treatment of MLTC-1 cells with 5×10^{-5}M and 7.5×10^{-5}M cinnamaldehyde for 24h significantly

increased testosterone secretion compared to 0, 10×10^{-5}, 20×10^{-5} and 40×10^{-5}M CMA concentrations ($P<0.05$; Fig. 5A); differences in testosterone secretion between former two and among the latter four CMA concentrations were statistically non-significant. At 48h, 7.5×10^{-5}, 10×10^{-5} and 20×10^{-5}M CMA treatment significantly increased ($P<0.05$) testosterone secretion by MLTC-1 cells compared to control (0×10^{-5}M) CMA (Fig. 5B). Differences in testosterone secretion by MLTC-1 cells at 48h among all 5 concentrations of CMA (5.0×10^{-5}M to 40×10^{-5}M) were statistically non-significant.

Effect of CMA treatment on MLTC-1 cell cycle: Flow cytometry and fluorescence quantitative PCR were used to analyze the effects of 5×10^{-5}M cinnamaldehyde treatment on the cell cycle and expression of cyclins in MLTC-1 cells. The results of flow cytometry showed that treatment with 5×10^{-5}M cinnamaldehyde significantly increased ($P<0.05$) the proportion of cells in the S phase, while reduced the proportion of cells in the G1 phase, with no effect on the proportion of cells in the G2 phase (Fig. 6A). The results of fluorescence quantitative PCR indicated that treatment with 5×10^{-5}M cinnamaldehyde upregulated the expression of cell cycle proteins Cyclin-A1 and Cyclin-B1 ($P<0.05$), with no impact on the expression of Cyclin-D2 (Fig. 6B).

Effect of cinnamaldehyde treatment on steroid hormone synthase in MLTC-1 cells: To investigate how cinnamaldehyde affects testosterone secretion, steroidogenic enzymes expression in MLTC-1 cells following cinnamaldehyde treatment was assessed using RT-qPCR and WB. The RT-qPCR results showed that 5×10^{-5}M cinnamaldehyde treatment significantly increased ($P<0.05$) the mRNA levels of CYP17A1 compared to control. In contrast, the mRNA levels of CYP11A1 and 3β-HSD showed a significant decrease, while there was no effect on StAR levels (Fig. 7A). Furthermore, WB analysis revealed that 5×10^{-5}M cinnamaldehyde treatment significantly ($P<0.05$) upregulated the expression of CYP17A1, CYP11A1, and 3β-HSD proteins. In contrast, the expression of StAR protein was reduced compared to control (Fig. 7B).

Effect of CMA on the expression of steroid hormone synthesis nuclear receptors in MLTC-1 cells: The expression of steroid hormone synthesis nuclear receptors and the activity of their promoters using RT-qPCR and dual Luciferase reporter assays was also monitored in this study. The results indicated that treatment with 5×10^{-5}M cinnamaldehyde increased SF-1 and SHP mRNA levels, while Nur-77 mRNA levels were decreased compared to control ($P<0.05$; Fig. 8A). Dual-luciferase reporter plasmids for the Nur-77 and SF-1 promoters were successfully constructed (Fig. 8B). Using the dual-luciferase reporter assay, the effects of the 5×10^{-5}M cinnamaldehyde treatment on Nur-77 and SF-1 promoter activity were also evaluated (Fig. 8C). The 5×10^{-5}M cinnamaldehyde treatment enhanced SF-1 promoter activity but did not affect Nur-77 promoter activity when compared with control group.

DISCUSSION

Testicular interstitial cells are the main cells that secrete testosterone in male mammals (Zirkin and Papadopoulos, 2018). In recent years, various studies have focused on the role that traditional Chinese medicinal extracts play in regulating physiological processes, such as hormone secretion (Liu *et al.*, 2025; Wang *et al.*, 2025). Cinnamaldehyde is an active compound of the traditional Chinese medicine, *Cinnamomum cinnamon* (Dou *et al.*, 2018; Gopalakrishnan *et al.*, 2023). The purpose of this research was to investigate the role of cinnamaldehyde (CMA) in the regulation of hormone synthesis. In vivo trial results showed that treatment with 25mg/kg CMA

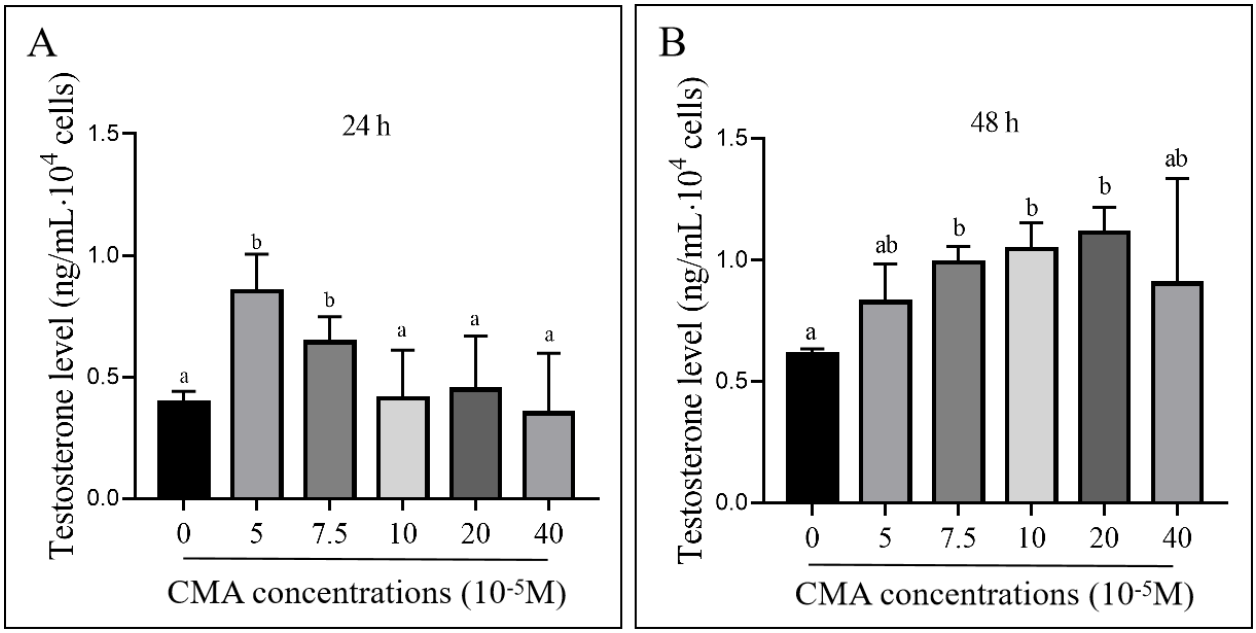

Fig. 5: CMA-modulated testosterone production in MLTC-1 cells. A): Testosterone concentration in the supernatant of each group after 24h treatment. B): Testosterone concentration in the supernatant of each group after 48h treatment. Triplicate biological replicates (mean±SEM); different letters indicate significant intergroup variations within 24h and 48h ($P<0.05$).

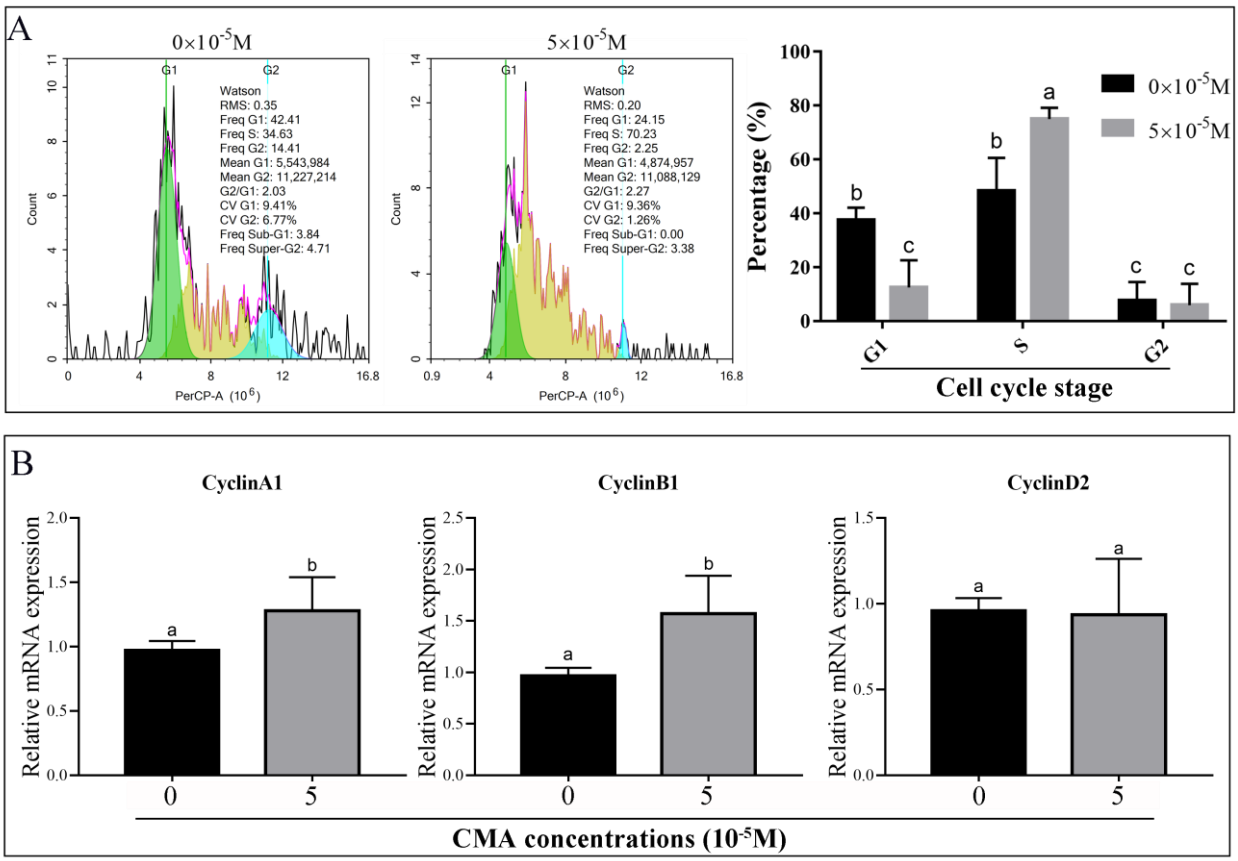

Fig. 6: CMA-modulated cell cycle dynamics and cyclin regulatory network in MLTC-1. A): FACS analysis with quantitative profiling (representative plots from triplicate experiments). B): Cyclin mRNA quantification: RT-qPCR of A1/B1/D2 transcripts (GAPDH endogenous control). Data (mean±SEM) with different superscript letters denote significant intergroup differences for each parameter (P<0.05).

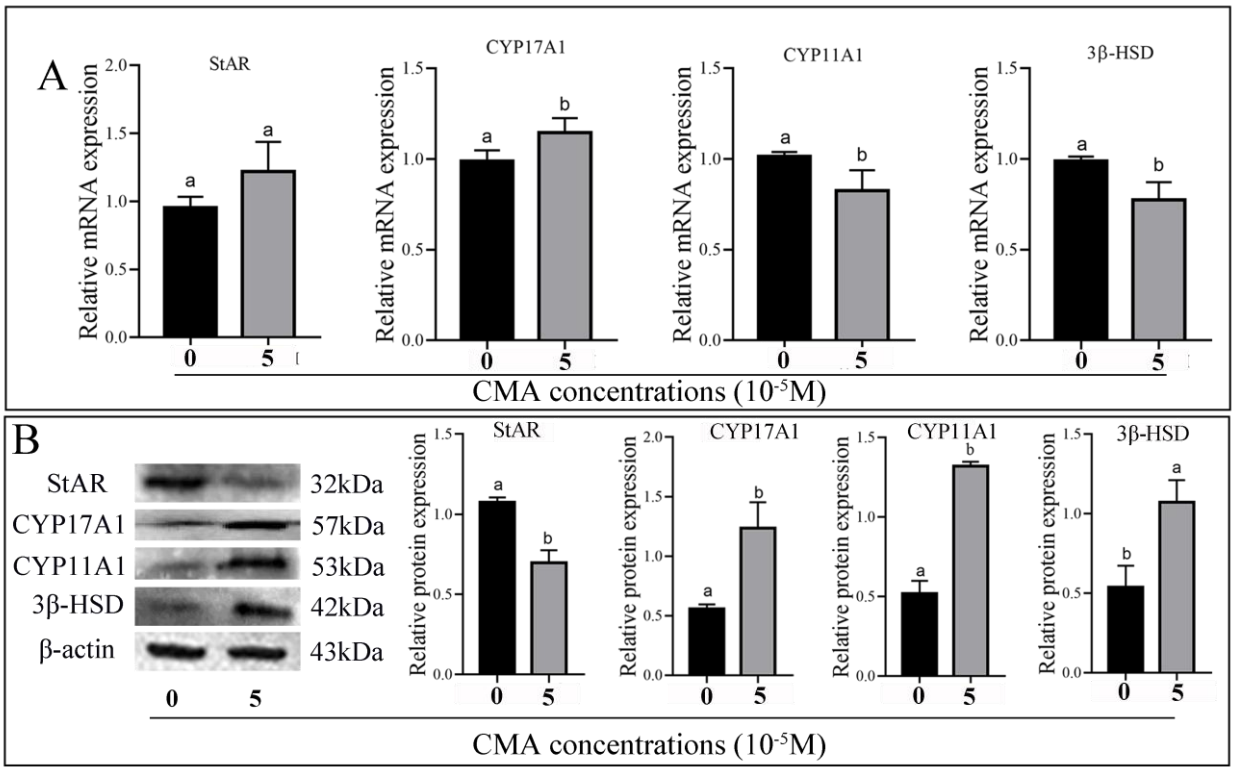

Fig. 7: CMA-modulated steroidogenic enzyme regulation in MLTC-1. A): Steroidogenic transcripts: RT-qPCR analysis of StAR/CYP17A1/CYP11A1/3β-HSD mRNA (GAPDH endogenous control). B): Protein quantification: Immunoblotting of corresponding enzymes (β-actin loading control). Triplicate biological replicates (mean±SEM); different superscript letters indicate intergroup significant difference for each parameter (P<0.05).

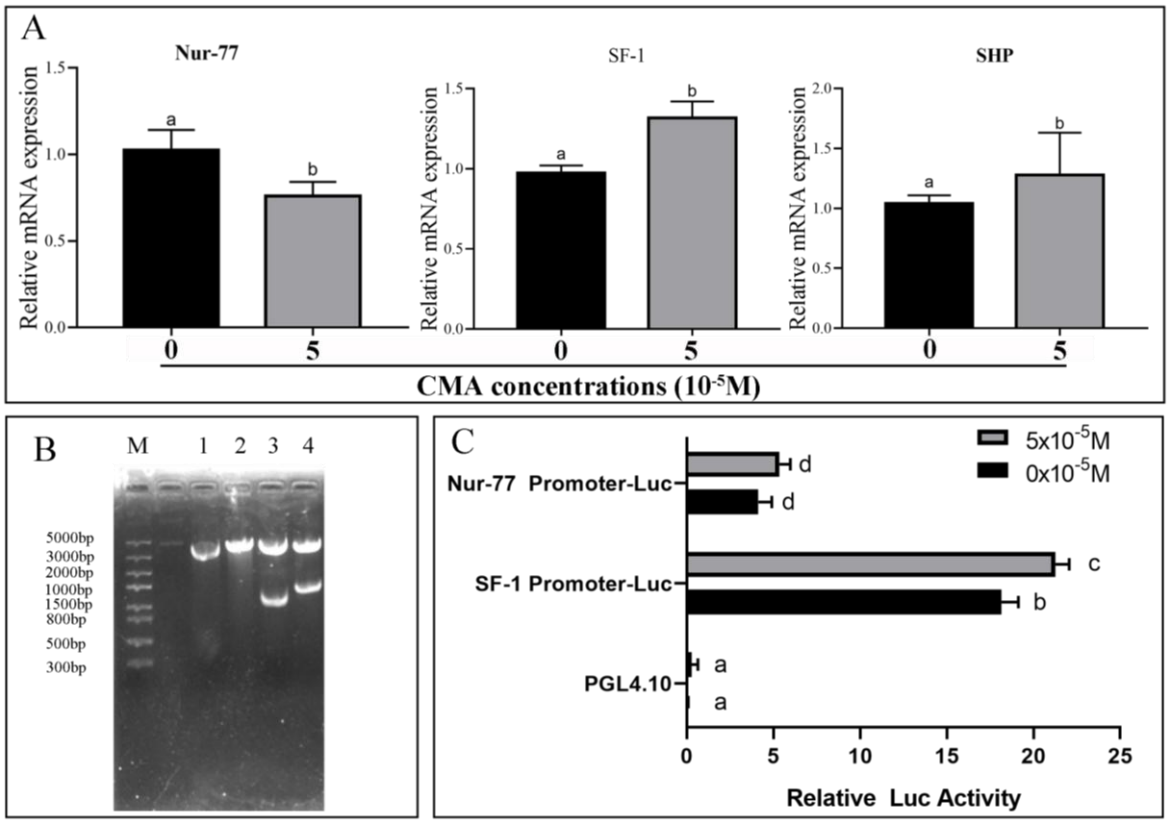

Fig. 8: CMA-regulated nuclear receptor circuitry in steroidogenic MLTC-1. A): Transcript profiling: Nur-77/SF-1/SHP mRNA quantification (RT-qPCR; GAPDH endogenous control). B): Construct validation: Restriction mapping of pNur-77-Luc/pSF-1-Luc reporters (M: DL5000 marker; lanes 3-4: validated constructs). C): Promoter modulation: Dual-luciferase assays of nuclear receptor activity. Triplicate experiments (mean±SEM); different letters denote intergroup significant difference for each parameter (P<0.05).

significantly increased serum testosterone concentrations, suggesting that CMA stimulates the synthesis and secretion of testosterone in mice. In order to further investigate the mechanism of CMA regulating testosterone secretion, MLTC-1 cells were treated with CMA. The results indicated that treatment of MLTC-1 cells with 5×10^{-5}M CMA for 24h increased testosterone secretion from these cells, suggesting that CMA may effectively convert cholesterol to testosterone in mouse Leydig cells. The control of testosterone secretion is a complex process involving many enzymes and pathways related to testosterone synthesis and secretion. Our results suggest that cinnamon may play an important regulatory role in testosterone secretion, but there is lack of information regarding the pathway or mechanism of this action.

During the conversion of cholesterol to steroid hormones by testicular interstitial cells, StAR first transports cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (Gomez-Sanchez and Gomez-Sanchez, 2024). Cholesterol is then converted to pregnenolone through catalytic activity of the mitochondrial CYP11A1 enzyme, and pregnenolone is catalyzed to progesterone by 3β-HSD on the smooth endoplasmic reticulum (Kraemer, 2007). Immature testicular mesenchymal stromal cells do not secrete testosterone because the produced progesterone is mainly catalyzed by CYP17A1 to androstenedione. Mature testicular interstitial cells begin to secrete testosterone, and androstenedione is converted to testosterone by the catalytic action of 17β-HSD. CYP17A1 and CYP11A1, both belong to the P450 enzymes family and play crucial roles in catalyzing the synthesis of steroid hormones. CYP11A1 and StAR control the amount of synthesized androgen precursors and serve as the two most critical rate-limiting enzymes catalyzing the conversion of cholesterol to testosterone in testicular interstitial cells.

It has been shown that CYP11A1 and StAR enzyme expression (both mRNA and protein levels) and activity positively correlate with testosterone synthesis in Leydig cells (Gomez-Sanchez and Gomez-Sanchez, 2024). ELISA assays identified CMA as a potent stimulator of testosterone secretion in these cells. Our RT-qPCR and Western blot analysis revealed that CMA upregulates key steroidogenic proteins (CYP17A1 and CYP11A1), indicating its ability to enhance testosterone production through modulation of enzymatic pathways.

The nuclear receptor is a main regulator of gene expression related to testosterone synthesis (Taniguchi *et al.*, 2009; Wu *et al.*, 2024). Nur77 and SF-1 modulate testosterone production by directly binding to promoter regions of steroidogenic enzymes. For example, SF-1 enhances transcription of 3β-HSD, CYP11A1, StAR, and CYP17A1 in Leydig cells through CREB-dependent activation (Dube *et al.*, 2009; Azmahani *et al.*, 2014). Especially, Nur-77 and SF-1 exhibit faster StAR induction than cAMP-mediated pathways. In contrast, the nuclear receptor SHP acts as a critical suppressor of steroidogenesis (Di-Luoffo *et al.*, 2015).

Luteinizing hormone (LH) regulates steroidogenesis by upregulating Nur-77, which subsequently targets genes including CYP17A1, 21-hydroxylase, and 20α-HSD

through direct promoter binding (Zhang and Mellon, 1997; Martin *et al.*, 2008). Our RT-qPCR analysis revealed that CMA treatment upregulates SF-1 expression in mouse Leydig cells. Given the dual role of Nur-77 in proliferation and apoptosis (Yu *et al.*, 2020), we propose that CMA modulates steroidogenesis via coordinated activation of SF-1 and Nur-77 promoters. Promoter activity assays confirmed significant cinnamaldehyde-induced enhancement of both nuclear receptors, suggesting their involvement in downstream steroidogenic gene regulation. Based on the above results, it is fair to believe that CMA can regulate the expression of nuclear receptors by enhancing the activity of their promoters and then promoting the expression of enzymes related to steroid hormone synthesis and ultimately regulating testosterone secretion in Leydig cells.

Cell cycle proteins (cyclins) regulate cell cycle progression through cyclic synthesis and degradation in response to the time-phase shift of the cell. Cyclins include cyclin-D, cyclin-A, cyclin-E and cyclin B. Cyclin-D is mainly synthesized in the G1 phase and disappears in the S phase, and is responsible for activating the Cdk-2, Cdk-4, and Cdk-6 proteins in the G1 phase. Cyclin-A and cyclin-E are S phase proteins that begin to disappear at the onset of the G2 phase, both of them can activate Cdk-2 proteins in G1/S phase. Cyclin-B is a G2/M phase protein that can work in conjunction with cyclin-A to regulate the activity of Cdk-1 during the G2/M phase (Johansson and Clarke, 2022; Wang *et al.*, 2024).

Cyclin-A orchestrates replication through Cyclin-E while preventing re-initiation via Cdc-6 phosphorylation, and terminates S-phase by inactivating E2F (Yang *et al.*, 1999). Cyclin-B1 expression, driven by FoxM1 and B-Myb (Zhu *et al.*, 2004), peaks at G2/M through Cyclin A-Cdk2 activation (Vigneron *et al.*, 2018), exhibiting cytoplasmic-nuclear shuttling during mitosis (Alvarez-Fernandez and Malumbres, 2014). In the present study, cinnamaldehyde (5×10^{-5}M) significantly altered murine Leydig cell cycle distribution, decreasing G1-phase populations by 18% ($P<0.05$) while increasing S-phase cells by 23% ($P<0.01$). This was accompanied by 2.7-fold (cyclin-A1) and 3.1-fold (cyclin-B1) mRNA upregulation versus controls, suggesting cinnamaldehyde modulates testicular interstitial cell proliferation through cyclin-mediated mechanisms.

Conclusions: Steroid reproductive hormones play an important role in regulating the reproductive functions of mammals. Cinnamaldehyde is the main component extracted from the plant cinnamon. This study indicates that CMA regulates the steroid hormone synthesis enzymes expression by modulating the activity of Leydig cell nuclear receptor promoter, ultimately affecting the secretion of testosterone by Leydig cells. The present study provides experimental evidence for revealing the process by which CMA regulates reproductive hormone secretion and enhances the application value of cinnamon.

Conflict of interest: The authors confirm that no conflicts of interest exist.

Authors contribution: Qingrui Meng and Xiaoyu Huang carried out investigations, methodology, formal analysis, data curation and visualization. Lei Wang was involved in conceptualization, investigation and funding acquisition. Congguang Cai, Yuxin Guo, Yang Yang, Guangwen Yin, Muhammad Mohsin and Rao Zahid Abbas were responsible for writing, review, editing and validation of the manuscript. Dengfeng Wang supervised the study, while Guangwen Yin was responsible for project administration. All authors reviewed and approved final version of the manuscript.

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