

Research Article

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Sericin Improves Age-Related Copulatory Impairment in Rats Through Testosterone Enhancement and PI3K/Akt/Enos Signaling Modulation

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ABSTRACT

Purpose: Impaired sexual behavior associated with aging remains a significant global health concern. Studies are investigating natural compounds to identify effective interventions for counteracting this condition. The present study examined the effectiveness of sericin (Ser) on age-related sexual impairment in aged rats.

Methods: A total of 10 young adult and 40 aged male Wistar rats were used. The aged animals were categorized into four groups and received NS or Ser at doses of 200, 250, and 300 mg/kg for 30 consecutive days. Copulatory behavior was assessed to evaluate sexual function. At the end of the behavioral assessment, serum testosterone levels, penile p-PI3K/PI3K, p-Akt/Akt, p-eNOS/eNOS, and p-NF- κ B/NF- κ B ratios, oxidative stress markers (TAC, MDA, GPx, and SOD), as well as cGMP, NO, IL-6 and TNF- α levels were measured. Notably, biochemical evaluations (n = 6/group) and Western blotting (n = 3/group) were conducted on representative subsets.

Results: The results demonstrated that Ser improved copulatory behavior in aged animals. The enhancement in sexual behavior was associated with increased PI3K/Akt/eNOS signaling and restoration of the NO/cGMP pathway, elevated testosterone levels, reduced lipid peroxidation, increased TAC and antioxidant enzyme activities, as well as diminished penile levels of IL-6 and TNF- α in aged rats.

Conclusion: These findings suggest that the ability of Ser to attenuate oxidative stress and inflammation may represent a promising approach for restoring testosterone levels and PI3K/Akt/eNOS signaling, thereby alleviating age-related sexual dysfunction. Nevertheless, further studies are required to clarify the mechanisms and evaluate therapeutic potential of Ser in the management of sexual dysfunction associated with aging.

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Introduction

Sexual dysfunction comprises disturbances in desire, arousal, ejaculation, erection, or orgasm that interfere with sexual activity.¹ Erectile function depends on neural, vascular, endothelial, and hormonal mechanisms.²⁻⁴ Cavernosal structural defects, denervation, and endothelial or hormonal impairments induce erectile dysfunction (ED) and its associated behavioral consequences.^{2,3} Among these, endothelial dysfunction, marked by decreased nitric oxide (NO) bioavailability, impairs cavernosal hemodynamics and serves as a primary driver of ED.⁴ Aging is considered a major risk factor for ED, with the prevalence and severity of the condition increasing with advancing age.⁵ Evidence from the Massachusetts Male Aging Study indicated that nearly 26 out of every 1000 men experience ED annually. Moreover, the annual incidence of ED increases more than threefold, rising from 1.2% in men aged 40-49 years to 4.6% among those aged 60-69 years.⁶ Aging is associated with a vicious cycle of low-grade inflammation and oxidative stress⁷, which impairs NO signaling by diminishing NO synthesis and bioavailability.⁸ Additionally, age-related deficiencies in testosterone synthesis further exacerbate these disruptions in NO signaling.⁹ Consequently, older men experience reduced sexual desire and a higher frequency or severity of erectile difficulties, often requiring more time and stimulation to achieve an erection that is satisfactory for sexual performance.^{10,11} Currently, phosphodiesterase-5 inhibitors (PDE5Is), including tadalafil, sildenafil, and vardenafil, are prescribed as first-line treatments for ED in older men, with an estimated efficacy of only 65.7%.¹² However, their use may increase the risk of retinal defects and is contraindicated in older patients concurrently taking vasodilators such as nitrates, potentially restricting the clinical use of PDE5Is in this population.^{12,13} Based on this background, age-related sexual dysfunction should be given special attention as one of the primary medical problems among older adults, and the development of effective treatments for its management is of great importance.

Sericin (Ser) is a water-soluble silk protein composed of 18 amino acids, naturally associated with fibroin in the silk cocoon of *Bombyx mori* and accounts for approximately 30% of the cocoon mass.¹⁴ It is a bio- and immune-compatible globular protein exhibiting versatile properties including photo-protection, anti-tumor, anti-inflammatory, free radical stabilizing, apoptosis modulation activities.¹⁵⁻¹⁹ Therefore, it has gained attention for its utility in the pharmaceutical, cosmeceutical, biomaterials, and nutraceutical industries.¹⁵ Studies have demonstrated that Ser may mitigate age-related cellular and molecular changes in both *in vitro* and *in vivo* models. For instance, *in vitro* assays have shown that Ser can prevent skin aging within cell culture models.²⁰

Moreover, findings showed that Ser not only restored organ indices but also attenuated histological changes in renal, cerebral, and hepatic tissues of D-galactose-induced aging mice.¹⁸ Furthermore, our previous findings demonstrated the anti-aging potential of Ser by restoring cognitive function in a mouse model of accelerated aging.²¹ In all of these studies, the antioxidant properties of Ser are the common mechanism through which it mediates its anti-aging effects. However, its role in modulating other processes, such as inflammation and programmed cell death, should not be neglected.^{18,20,21} Importantly, the anti-inflammatory effect of Ser, reported in a wide range of experimental studies, represents a remarkable property that may contribute as significantly as its antioxidant activity, especially under conditions where oxidative stress and inflammation jointly disrupt biological functions.^{17,21,22}

Based on this premise, and considering that age-related sexual dysfunction may partly result from hormonal and vascular alterations induced by oxidative stress and inflammation, we hypothesized that treatment with Ser could improve copulatory behavior in naturally aged rats.

Materials and Methods

Animals and study design

A total of 40 aged male Wistar rats (18-20 months old), 10 young adult male Wistar rats (5-6 months old), and the same number of female rats (n=50, 5-6 months old) were used in this study. Aged rats were chosen as a natural model to mimic age-dependent copulatory dysfunction. Female rats were used as receptive stimuli for subsequent copulatory evaluations.

Rats were group-housed (5 per polypropylene cage) on wood-shaving bedding under regulated conditions ($24 \pm 2^\circ\text{C}$, $55 \pm 5\%$ relative humidity, and 12-h light/dark cycle) with free access to food pellets and water.

Aged Male rats were assigned into following groups (n = 10/ group); NS, Ser200, Ser250, and Ser300. Rats in both young and NS group received normal saline and remaining groups were given Ser (Lyph20180205, Xian Lyphar Biotech Co., China) at doses of 200, 250, or 300 mg/kg.

Ser doses were selected based on previous studies in various rat models, including Parkinson's²³ disease, underactive bladder²⁴, and diabetes^{25,26}, which employed the mentioned doses of Ser without evidence of behavioral abnormalities, systemic adverse effects, or mortality within this dose range.

Additionally, preclinical toxicological findings indicated that Ser was non-mutagenic, non-genotoxic and did not induce maternal, embryo-fetal, or teratogenicity.^{27,28} Furthermore, in both a 90-day repeated dose toxicity study in male and female rats²⁷ and a 10-day study in pregnant rats²⁸, the no-observed-adverse-effect level (NOAEL) was consistently established at 1000 mg/kg/day. Therefore, the doses used in experimental rat models (200-300 mg/kg) were below the reported toxicological NOAEL, suggesting an acceptable preliminary safety margin.

All solutions were freshly prepared daily prior to administration and were administered orally via gastric gavage at a constant volume of 10 mL/kg for 30 consecutive days (Time course of study was shown in Fig.1). In all procedures used in this study, NIH guidelines (Publication No. 85-23, revised 1985) were followed and approval was obtained from the Tabriz University of Medical Sciences (IR.TBZMED.AEC.1403.053).

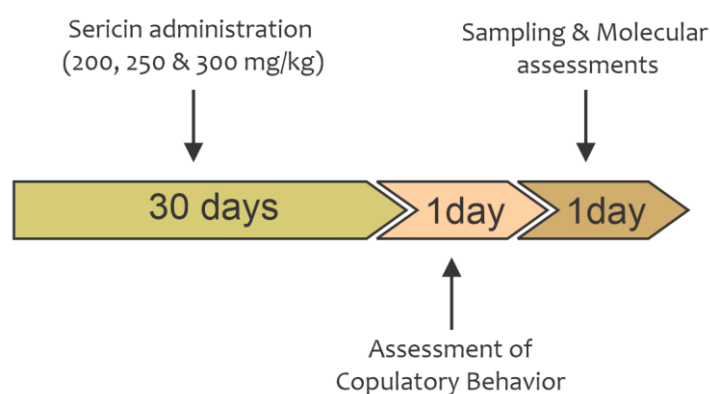


Figure 1. Time course of study.

Randomization and Blinding

we used publicly available platform (<https://www.graphpad.com/quickcalcs/randomize2/>) for randomization.

In all experimental phases, blinding was maintained to avoid any observer bias. Thus, all treatments and samples were coded by independent investigators. Procedures including behavioral testing, tissue harvesting, biochemical assays, and western blot densitometry were performed in a blinded manner. Finally, group identities were unblinded for the statistical analysis.

Preparation of receptive rats

To prepare receptive females, the rats underwent bilateral ovariectomy 45 days prior to copulatory behavior testing in the study groups. Briefly, under intraperitoneal anesthesia with ketamine (70 mg/kg) and xylazine (7 mg/kg), two incisions (~1 cm in length) were made through the back skin and the underlying dorsolateral muscles on each side. The peri-ovarian adipose pad was gently pulled out, the distal uterine horn was subsequently ligated, and the ovary was excised.²⁹

Receptivity confirmation

Sexual receptivity was verified 30 days post-ovariectomy and 15 days before their introduction to the experimental male rats. For this, ovariectomized rats were primed via subcutaneous injections of estradiol benzoate (Aburaihan Pharmaceutical Co., Tehran, Iran, 0.1 mg/kg) and progesterone (Iran Hormone Co., Iran, 2 mg/kg), administered 48 h and 4 h before behavioral testing, respectively.¹³ They were then introduced to a sexually experienced male rat not belonging to the experimental cohort. Lordosis responses were screened across 10 mounts and scored on a 0-3 scale. The lordosis quotient (LQ) was calculated as the percentage of mounts eliciting a score of 2 or 3, excluding marginal lordosis (score 1), and females achieving an $LQ \geq 60\%$ were confirmed to be receptive.³⁰ A 15-day hormone-free washout period was included between the confirmation session and the experimental test to ensure complete hormonal clearance.³¹

Assessment of Sexual performance in male rats

To assess copulatory behaviors 24 h following the final Ser administration (day 31), each male rat was placed in an observation chamber (30 × 30 × 30 cm) approximately 5 min before pairing. Sexually receptive females, primed with sequential estradiol benzoate and progesterone as described above, were randomly introduced to males at a 1:1 ratio. Copulatory behavior was observed for a 30-min period, with each stimulus female used only once.

The following parameters were measured: mount latency (ML; the time elapsed from the introduction of the female rat to the first mount by the male rat), intromission latency (IL; the time elapsed from the introduction of the female rat to the first mount resulting in vaginal penetration), and ejaculation latency (EL; the time elapsed from the first intromission to ejaculation). In addition, mount frequency (MF; the number of mounts without vaginal penetration), intromission frequency (IF; the number of successful vaginal penetration), and ejaculation frequency (EF; the number of ejaculations observed during the test session) were recorded.¹³

Sampling and biochemical assays

24 h after copulatory experiment, rats were deeply anesthetized by intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg).³² Blood samples were collected by cardiac puncture between 10:00 and 12:00 h, falling within the broader diurnal acrophase of testosterone secretion.³³ Then, serum was obtained by centrifugation at 3000 rpm for 10 min. Penile tissues were subsequently isolated and stored at -70 °C for biochemical analysis. Animals were randomly selected from each group before outcome assessment, without reference to their behavioral or biochemical responses, and the assays were conducted with group allocation concealed from the investigators. Samples were obtained from a different animal; thus, the reported sample size represents biological, not technical, replicates.

Testosterone levels

Serum total testosterone levels were measured using a fluorescence immunoassay (FIA) kit (Cat. No. BT2211, Xiamen Biotime Biotechnology Co., Ltd., Xiamen, China) according to the manufacturer's instructions and analyzed using a Biotime FIA Analyzer (Xiamen Biotime Biotechnology Co., Ltd, Xiamen, China).

Oxidative stress markers

Penile samples were homogenized in ice-cold lysis buffer and then centrifuged (12000 rpm, 10 min, 4 °C) to obtain the supernatant. Then, supernatants were collected, and protein concentration was measured for normalization. The Nasdox and Nagpix kits (both from Navand Salamat Co., Urmia, Iran), were used to assay the activities of superoxide dismutase (SOD; at 405 nm; U/mg tissue protein) and glutathione peroxidase (GPx; at 340 nm; expressed as mU/mg tissue protein), respectively. The Naxifer kit (Navand Salamat Co., Urmia, Iran) was used to measure total antioxidant capacity (TAC) based on the FRAP method at 593 nm, and the results were expressed as mmol Fe²⁺/mg tissue protein. Malondialdehyde (MDA) levels, determining lipid peroxidation, were assayed using a TBARS-based assay kit (Teb Pazhouhan Razi Co., Tehran, Iran) at 530 nm and expressed as μmol/mg protein.

NO and cyclic guanosine monophosphate (cGMP) levels

NO levels were measured using the Natrix kit (Navand Salamat Co., Urmia, Iran) based on the Griess reaction, with indirect quantification via nitrite/nitrate and spectrophotometric detection of an azo dye at 570 nm and reported as μm/mg tissue protein.

cGMP levels were estimated by a competitive ELISA kit (FineTest Co., China) and expressed as pmol/mg tissue protein. The absorbance of the final color reaction product was read at 450 nm and concentrations were calculated from a standard curve.

Inflammatory cytokines

IL-6 and TNF-α concentration were measured in penile tissues using ELISA kits (Karmania Pars Gene, Rafsanjan, Iran; KPG-RIL6 and KPG-TNF-α-48, respectively) according to the manufacturer's protocol. The absorbance of samples was read at 450 nm and concentrations were calculated from standard curves.

Western blotting

To conduct Western blot analysis, tissue samples were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail. Total protein concentration was measured using Bradford's method. Equal amounts of protein were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with PBS containing 5% non-fat dry milk and 0.05% Tween-20 for 1h at room temperature. After washing, membranes were incubated overnight at 4 °C with primary antibodies including PI3 kinase p85 (PI3k; 1:1000; ab40755), phospho-PI3 kinase p85α (Tyr607) (p-PI3k; 1:1000; ab235266), Akt (1:500, ab8805), phospho-Akt (p-Akt; p1:500; ab38449), eNOS (1:1000, ab300071), NF-κB p65 (1:1000, ab16502), NF-κB p65 (phospho S536) (p-NF-κB; 1:1000; ab76302), and β-actin (1:2500, ab8227) all from Abcam (Cambridge, UK) while phospho-eNOS (Ser1177) (p-eNOS; 1:1000; GTX129058) was obtained from GeneTex Inc., (CA, USA). Membranes were washed with TBST (3 times) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:10000, ab6721). Immunoreactive bands

were visualized using an enhanced chemiluminescence (ECL) detection kit, and band intensities were quantified using ImageJ software (NIH, USA). Notably, PVDF membrane was re-probed for multiple target proteins, β -actin was first detected as the loading control. The membrane was subsequently stripped between successive probing steps using a mild stripping buffer containing 1.5% (w/v) glycine, 0.1% (w/v) SDS, and 1% (v/v) Tween-20, adjusted to pH 2.2. Following stripping, the membranes were thoroughly washed with TBST and re-blocked before incubation with the next primary antibody. The target proteins were detected sequentially, with phosphorylated forms detected before their corresponding total proteins in the following order: p-PI3K $\rightarrow$ PI3K $\rightarrow$ p-Akt $\rightarrow$ Akt $\rightarrow$ p-eNOS $\rightarrow$ eNOS $\rightarrow$ p-NF- κ B $\rightarrow$ NF- κ B p65. After each stripping step, the membrane was assessed for residual chemiluminescent signal by applying the ECL detection reagent in the absence of primary and secondary antibodies. No detectable chemiluminescent signal was observed before proceeding with the subsequent probing step. Protein expression levels were normalized to β -actin as a loading control.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 10.4.1 (GraphPad Software Inc., La Jolla, CA, USA). Continuous parametric variables were analyzed using one-way ANOVA followed by Tukey's post-hoc test and are presented as mean $\pm$ SEM. Behavioral count outcomes (MF, IF, and EF) were assessed using the non-parametric Kruskal-Wallis test followed by Dunn's post-hoc test for pairwise comparisons. For EL, animals failing to ejaculate within the 30-min testing period were right-censored at 30 min. Differences in EL distributions among groups were evaluated using the Kaplan-Meier method with the log-rank (Mantel-Cox) test, followed by planned pairwise comparisons. A P-value < 0.05 was considered statistically significant.

Results

Copulatory behaviors

For frequencies (Fig. 2A-C), the non-parametric Kruskal-Wallis test revealed significant overall differences among the groups for MF ($H(4) = 20.0$, $P = 0.0005$; Fig. 2A), IF ($H(4) = 27.2$, $P < 0.0001$; Fig. 2B), and EF ($H(4) = 24.8$, $P < 0.0001$; Fig. 2C). Dunn's post-hoc multiple comparisons indicated that NS- treated aged rats exhibited significantly lower MF ($P < 0.001$), IF ($P < 0.001$), and EF ($P < 0.01$) compared with young group. Treatment with Ser reversed these behavioral decrements, with the 300 mg/kg dose producing significant increases in MF ($P < 0.05$), IF ($P < 0.05$), and EF ($P < 0.05$) compared with aged rats in NS group. For latency parameters (ML and IL), one-way ANOVA demonstrated significant differences across groups for ML ($F(4, 45) = 28.8$, $P < 0.0001$; Fig. 2D) and IL ($F(4, 45) = 17.6$, $P < 0.0001$; Fig. 2E). Post-hoc Tukey's tests confirmed that aged rats had significantly prolonged ML ($P < 0.001$) and IL ($P < 0.001$) compared with young controls. However, Ser (250 and 300 mg/kg) significantly shortened both ML (at least $P < 0.01$ for both doses) and IL (at least $P < 0.05$ for both doses). (Fig. 2D-E).

Furthermore, Kaplan-Meier analysis of EL, with non-responders (non-ejaculating rats) right-censored at 30 min, revealed significant differences in EL among groups (log-rank test, $\chi^2(4) = 81.50$, $P < 0.0001$). Pairwise log-rank comparisons demonstrated a marked prolongation of EL in aged rats compared to young controls ($P < 0.0001$). Treatment with Ser at 250 mg/kg ($P = 0.0202$) and 300 mg/kg ($P = 0.0043$) significantly shortened EL relative to the NS-received aged rats, whereas the 200 mg/kg dose showed no significant effect ($P = 0.7849$). Also, censored observations (non-ejaculating rats) were present in the Aged, Ser200 and Ser250 groups (Fig. 2F).

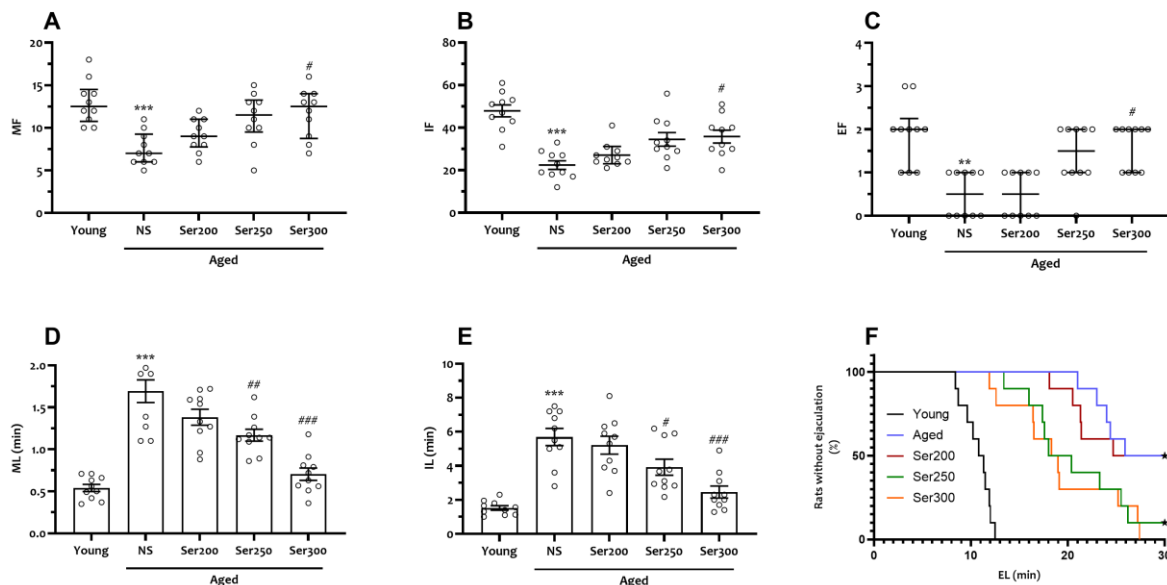


Figure 2. Effects of Ser on copulatory behavior parameters in aged rats. (A-C) show frequency measures; (A) MF, (B) IF and (C) EF. Data are presented as individual values with median and interquartile range (IQR) with $n = 10$ /group and were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test. (D-E) show latency parameters; (D) ML and (E) IL. Data are expressed as mean $\pm$ SEM with $n = 10$ per group and were analyzed by one-way ANOVA followed by Tukey's post-hoc test. (F) The Kaplan-Meier survival curves depicting EL distributions over a 30-min test period ($n = 10$ /group). Curves illustrate the percentage of rats without ejaculation. Non-responders were right-censored at 30 min and Data were analyzed using the log-rank (Mantel-Cox) test. Star symbols at 30 min indicate non-ejaculating rats during the observation period, plotted at 50% for overlapping Aged and Ser200 groups and at 10% for the Ser250 group. ** $p < 0.01$ and *** $p < 0.001$ vs. Young; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. NS-received aged rats. [MF, Mount frequency; IF, Intromission frequency; EF, Ejaculation frequency; ML, Mount latency; IL, Intromission latency; EL, Ejaculation latency; NS; Normal saline; Ser200, Ser250, and Ser300: 200, 250, and 300 mg/kg of sericin].

Testosterone levels

Significant differences in serum testosterone levels were observed among the study groups ($F(4,25) = 71.5$, $p < 0.0001$) according to one-way ANOVA. Post-hoc analysis revealed that, compared with the young group, aged rats in the NS group exhibited significantly lower testosterone levels ($p < 0.001$; Fig. 3), indicating marked age-related suppression of testosterone production. In contrast, Ser administration (250 and 300 mg/kg) significantly increased testosterone levels relative to NS-treated aged rats ($p < 0.001$ for both doses).

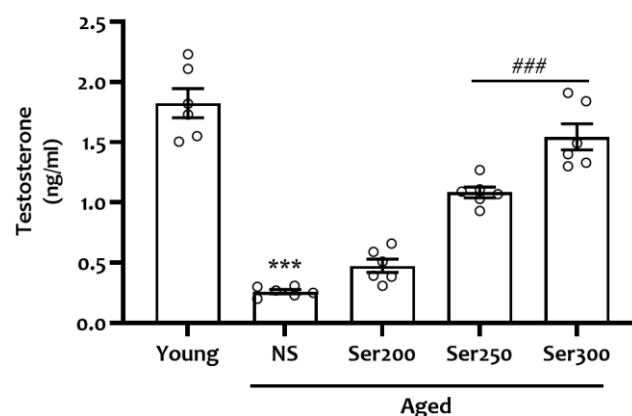


Figure 3. The Effects of Ser on serum testosterone levels of study groups. Values are shown as mean $\pm$ SEM with $n = 6$ /group and were analyzed by one-way ANOVA followed by Tukey's post-hoc test. *** $p < 0.001$ vs. young group, ### $p < 0.001$ vs. NS-treated aged group. [NS; Normal saline; Ser200, Ser250, and Ser300: 200, 250, and 300 mg/kg of sericin].

PI3K/Akt/eNOS axis and NO-cGMP signaling pathway

As determined by one-way ANOVA, significant differences were observed among the groups for p-PI3k/PI3k ($F(4,10) = 8.41$, $p = 0.0031$), p-Akt/Akt ($F(4,10) = 66.5$, $p < 0.0001$), p-eNOS/eNOS ($F(4,10) = 19.2$, $p = 0.0001$), NO ($F(4,25) = 45.7$, $p < 0.0001$), and cGMP ($F(4,25) = 23.4$, $p < 0.0001$).

Post-hoc analysis showed significantly lower p-PI3k/PI3k ($p < 0.01$; Fig. 4A and F), p-Akt/Akt ($p < 0.001$; Fig. 4B and F), and p-eNOS/eNOS ($p < 0.001$; Fig. 4C and F) ratios, as well as reduced NO ($p < 0.001$; Fig. 4D) and cGMP levels ($p < 0.001$; Fig. 4E) in aged rats compared with young animals. Administration of Ser at the dose of 200 mg/kg significantly increased p-Akt/Akt ratio ($p < 0.001$), with no significant changes in other components of the pathway compared with NS-treated aged rats. In contrast, Ser (250 and 300 mg/kg) significantly increased p-PI3K/PI3K (at least $p < 0.05$ for both doses), p-Akt/Akt ($p < 0.001$ for both doses), and p-eNOS/eNOS (at least $p < 0.05$ for both doses), as well as NO (at least $p < 0.01$ for both doses) and cGMP levels (at least $p < 0.01$ for both doses), relative to aged rats in the NS group.

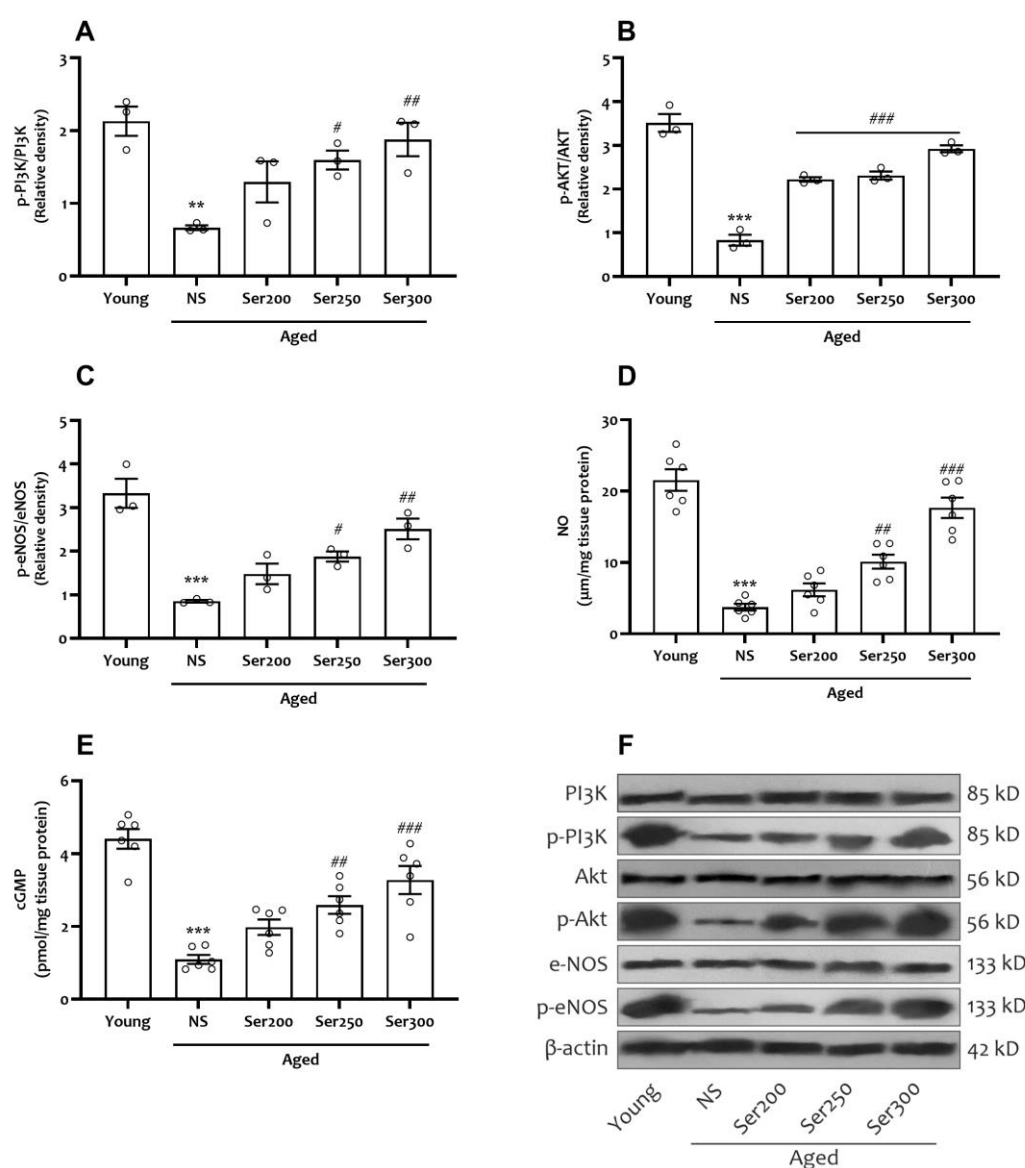


Figure 4. The Effects of Ser on penile levels of (A) p-PI3k/PI3k, (B) p-Akt/Akt, (C) p-eNOS/eNOS, (D) NO, and (E) cGMP. (F) Representative blot images. Values are shown as mean $\pm$ SEM with $n = 3$ /group for Western blot and $n = 6$ /group for ELISA analyses. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test. ** $p < 0.01$ and *** $p < 0.001$ vs. young group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. NS-received aged group. [NS; Normal saline; Ser200, Ser250, and Ser300: 200, 250, and 300 mg/kg of sericin].

Oxidative stress

One-way ANOVA revealed significant effects of treatment on MDA levels ($F(4,25) = 18.7$, $p < 0.0001$), GPx activity ($F(4,25) = 21.2$, $p < 0.0001$), SOD activity ($F(4,25) = 45.4$, $p < 0.0001$), and TAC ($F(4,25) = 58.3$, $p < 0.0001$) in penile tissue among the experimental groups.

Post-hoc analysis showed a significant increase in MDA levels ($p < 0.001$; Fig. 5A), along with marked reductions in GPx and SOD activities ($p < 0.001$ for both; Fig. 5B and C, respectively) and TAC ($p < 0.001$; Fig. 5D) in aged rats compared with young controls. In contrast, compared with aged rats in the NS group, MDA levels were significantly reduced in the Ser250 and Ser300 groups (at least $p < 0.05$ for both doses). Also, while GPx activity was significantly increased only in the Ser300 group ($p < 0.001$), SOD activity was elevated in all Ser-treated groups (at least $p < 0.05$ across all doses), and TAC was significantly increased following Ser administration at 250 and 300 mg/kg (at least $p < 0.01$ for both groups).

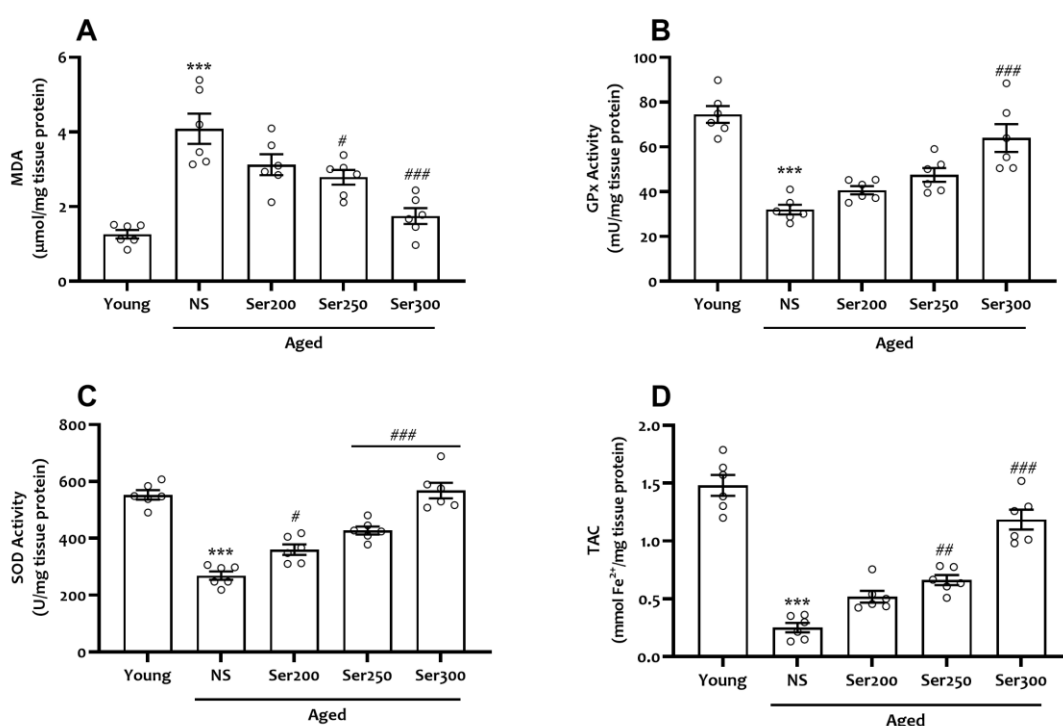


Figure 5. The Effects of Ser on levels of (A) MDA, (B) GPx activity (C) SOD activity, and (D) TAC in penile tissue of study groups. Values are shown as mean $\pm$ SEM with $n = 6$ /group and were analyzed by one-way ANOVA followed by Tukey's post-hoc test. *** $p < 0.001$ vs. young group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. NS group. [NS; Normal saline; Ser200, Ser250, and Ser300: 200, 250, and 300 mg/kg of sericin].

Inflammation

The one-way ANOVA revealed a significant effect of treatment on the p-NF κ B/NF κ B p65 ratio ($F(4,10) = 34.3$, $p < 0.0001$), as well as on IL-6 ($F(4,25) = 51.5$, $p < 0.0001$) and TNF- α ($F(4,25) = 58.5$, $p < 0.0001$) levels.

Post-hoc analysis showed that aged rats receiving NS exhibited a significant increase in the p-NF κ B/NF κ B p65 ratio ($p < 0.001$; Fig. 6A and D), as well as elevated IL-6 and TNF- α levels ($p < 0.001$ for both parameters; Fig. 6B and C, respectively), compared with young controls.

In contrast, administration of Ser (250 and 300 mg/kg) markedly reduced this ratio (at least $p < 0.05$ for both doses) and significantly decreased IL-6 (at least $p < 0.01$ for both doses) and TNF- α levels ($p < 0.001$ for both doses) in penile tissue of aged rats.

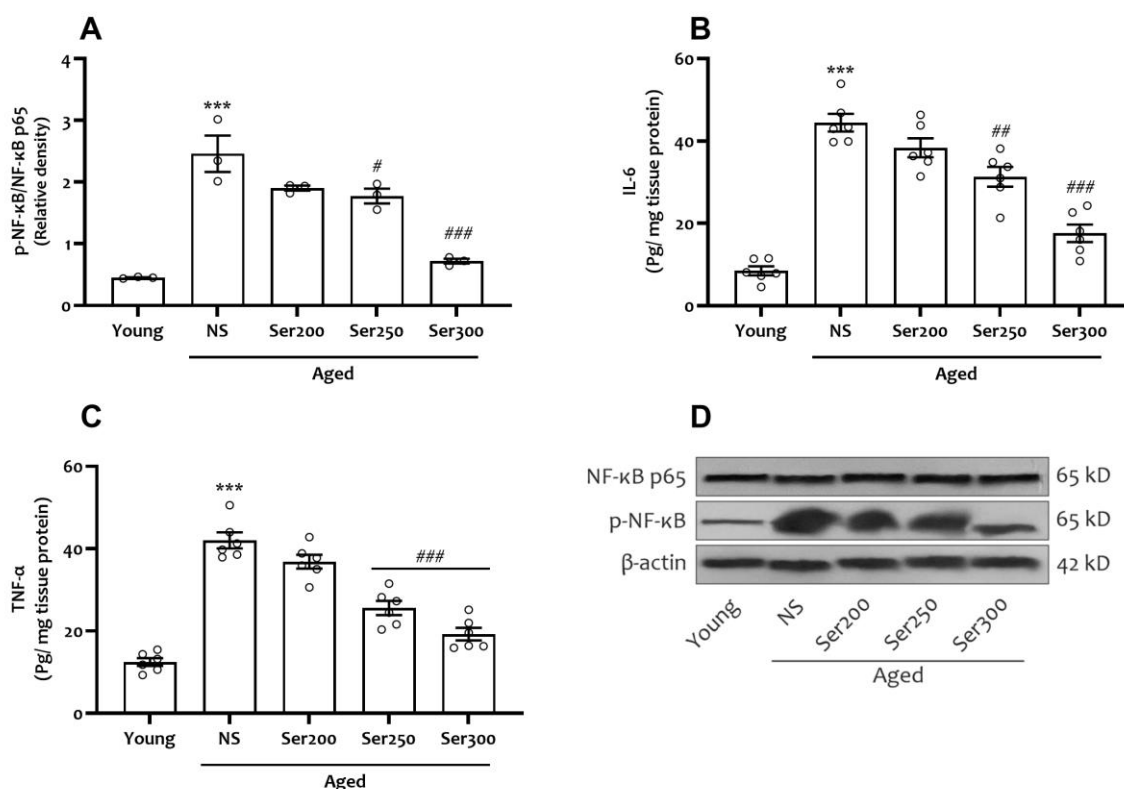


Figure 6. The Effects of Ser on penile protein levels of (A) p-NFκB/NFκB p65 ratio, (B) IL-6, (C) TNF-α. (D) Representative blot images. Values are shown as mean ± SEM with n = 3/group for Western blot and n = 6/group for ELISA analyses. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test. ****p* < 0.001 vs. young group, #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 vs. NS-treated aged group. [NS; Normal saline; Ser200, Ser250, and Ser300: 200, 250, and 300 mg/kg of sericin].

Discussion

In this study, chronic administration of Ser was associated with an attenuation of age-related copulatory dysfunction in male rats, partly through normalized serum testosterone levels, increased activation of the PI3K/Akt/eNOS and NO/cGMP signaling cascades, potentially via its antioxidant and anti-inflammatory properties.

Importantly, throughout the 30-day treatment, the animals tolerated the Ser well, exhibiting no mortality, gross behavioral distress, or overt signs of clinical morbidity.

In the context of copulatory behavior, it is well documented that copulatory latency measures, such as ML, IL, and EL, increase after 11 months of age, reaching peak levels at around 24 months.³⁴ However, changes in sexual behavior are not limited to latencies, and the frequencies of these behavioral parameters are also reduced in aged rats.³⁵ Consequently, these gradual age-related alterations, together with abnormalities in biochemical parameters and spermatozoa, contribute to a decline in reproductive capacity.³⁴

Our behavioral assessment indicated that aged rats not only exhibited significant increases in mount, intromission, and ejaculation latencies, but also presented lower frequencies of these behaviors. In contrast, treatment with Ser markedly reduced these latencies and increased behavioral frequencies. To accurately interpret these behavioral outcomes, it is essential to distinguish between sexual motivation, motor and ejaculatory performance, endocrine support, and local penile vascular signaling, as each uniquely influences copulatory consequences.

Since ML and IL are inversely associated with sexual motivation in rodents, reductions in these latencies primarily reflect enhanced sexual motivation and arousability.^{13,36} Hence, the significantly shortened latencies, comparable

to those of young controls, observed in aged rats receiving Ser at doses of 250 and 300 mg/kg may reflect increased sexual arousability and motivation.

Distinct from pure motivation, Mount and intromission frequencies reflect motor capability³⁷, copulatory efficiency, libido, and potency, serving as indices to assess ejaculatory reflex activation and general erectile function.^{36,38,39} Our results indicated that Ser treatment was associated with improvement in both sexual libido and potency in aged rats. Additionally, the ability of Ser to reduce EL and increase EF may enhance copulatory performance, whereas it has been suggested that compounds such as sildenafil, which mediate similar effects improves impaired copulatory performance in aged rats.¹³

Beyond central drive and motor performance, male sexual function and erectile integrity are closely linked to the serum testosterone levels and administration of testosterone has been shown to increase sexual libido, orgasm as well as ejaculation.⁴⁰ It has been documented that compounds containing phenolics and flavonoids may support circulating testosterone.³⁶ Given that Ser contains measurable amounts of phenolic and flavonoid derivatives¹⁸, the observed enhancement in copulatory behavior may be partially supported to its ability to elevate testosterone levels in aged rats.

In the context of peripheral penile function, testosterone not only maintains penile structural integrity but also facilitates erectile responses, which are correlated with healthy vascular function and NO signaling.^{41,42} penile erection in rats is testosterone-dependent, and evidence suggests that testosterone regulates the expression and activity of both endothelial (e-) and neuronal (n-) nitric oxide synthase (NOS), thereby contributing to the maintenance of physiological NO levels.⁴³ Our results showed that serum testosterone level was significantly reduced in aged rats, whereas Ser treatment (250 and 300 mg/kg) shifted them toward the values observed in young control rats.

Accumulating evidence suggests that inflammation and oxidative stress contribute to the decline in testosterone levels in aged rats.^{44,45} Consequently, reducing oxidative stress, modulating aromatase and melatonin levels, restoring testicular steroidogenic function, and correcting growth hormone/ insulin-like growth factor-1 axis dysfunction via regulation of growth hormone receptor expression and insulin-like growth factor-1 availability may all contribute to the observed improvement of circulating testosterone following Ser treatment.^{46,47}

At the local tissue level, normal sexual and erectile function requires the coordinated activity of neuronal and vascular systems. NO has a pivotal role in this process, produced via nNOS at autonomic nerve terminals to initiate tumescence and via eNOS in endothelial cells to sustain it.⁴⁸ Given the importance of these enzymes in the regulation of sexual function, conditions such as vascular aging, which contribute to reduced nNOS and eNOS expression, can lead to the development of ED.^{44,49} Physiologically, stimulants (whether psychogenic or reflexive) depolarize the cavernous nerves, leading to the release of NO. This NO then diffuses into the smooth muscle of the corpus cavernosum, where it stimulates cGMP production, mediating relaxation and thereby increasing penile blood flow and enlargement of corpus cavernosum.^{48,50} Subsequently, The resulting increase in shear stress further promotes NO release via activation of the endothelial PI3K/Akt/eNOS pathway.⁴⁸

The catalytic ability of eNOS is heavily depends on phosphorylation at Ser1177, by the enzymatic activity of PI3K/Akt axis.⁵¹ Activated PI3K promotes the generation of PIP3, which binds to Akt. This induces the conformational change and phosphorylation required to enhance Akt kinase activity, in turn activating eNOS via phosphorylation.⁵²

Our results showed a marked reduction in p-PI3K/ PI3K, p-Akt/Akt, and p-eNOS/eNOS ratios along with lower NO and cGMP levels in penile tissue of aged rats. Chronic administration of Ser for 30 days markedly increased these protein ratios. These findings are in line with reports demonstrating that penile PI3K/Akt/eNOS and

NO/cGMP pathways decline in aged rats⁵³⁻⁵⁶, and are impaired under conditions such as diabetes mellitus, metabolic syndrome, and cavernous nerve injury.⁵⁷⁻⁶⁰

The PI3K/Akt/eNOS and NO/cGMP cascades appear particularly vulnerable to age-related oxidative stress and inflammation, which are associated with reduced NO bioavailability and subsequent sexual dysfunction.⁴⁹ For instance, intracavernosal pressure, as an index of erectile function, and penile cGMP concentrations significantly declined in 18- to 22-month-old rats, paralleling impairments in copulatory parameters.¹³ Moreover, eNOS activity is negatively influenced by inflammation-driven oxidative stress via eNOS uncoupling. This phenomenon occurs due to the depletion of tetrahydrobiopterin levels, which acts as an essential eNOS co-factor.⁶¹ This uncoupling not only disrupts normal endothelial function but also exacerbates both oxidative and inflammatory damages.^{61,62} It seems that, regimens that help to restore these pathways have been associated with improvements in sexual function.^{56,57,60} Given the antioxidant and anti-inflammatory properties of Ser, the observed effects on the PI3K/Akt/eNOS axis and NO-cGMP signaling may link, at least in part, to these protective mechanisms. However, direct mechanistic causality remains to be validated through targeted pharmacological blockade or rescue studies.

Oxidative stress, primarily driven by cellular exposure to excessive levels of reactive molecules such as reactive oxygen species (ROS), originates from an imbalance between oxidants and the antioxidant defense system. In the vasculature, ROS are primarily generated by the mitochondrial electron transport chain, NADPH oxidases, cyclooxygenases, xanthine oxidase, and uncoupled eNOS.⁶³ During aging, the diminished capacity and activity of antioxidant defense systems primarily lead to lipid peroxidation, chronic inflammation, DNA damage, and the activation of apoptotic pathways.^{7,64,65} oxidative stress impairs cavernosum nerves and endothelium machineries, leading to the to the endothelial dysfunction and subsequent appearance of ED.⁶⁴⁻⁶⁶ In our study, increased level of lipid peroxidation (indicated by MDA) was associated with a marked reduction of TAC and reduced activities of both SOD and GPx enzymes in penile tissue of aged rats.

Previous findings in aged rats demonstrated a compromised antioxidant defense system in the corpus cavernosum, indicated by reduced SOD activity and GSH content, accompanied by increased superoxide anion and MDA levels.^{65,67}

Chronic Ser treatment was associated with a restoration of antioxidant markers (SOD, GPx, and TAC). The ability of Ser to attenuate lipid peroxidation while supporting endogenous antioxidant systems has been reported in peripheral and central tissues across various experimental models.^{16,18,21,22,68,69}

Ser can exert antioxidant activity via various mechanisms. Its high content of polar amino acids (mainly serine and threonine) bearing hydroxyl groups enables the chelation of trace metals, including iron and copper.¹⁸ Molecular docking findings have shown that Ser-derived peptides inhibit ROS-generating enzymes.⁷⁰ Additionally, in vitro studies have shown its ability to scavenge DPPH free radicals^{18,70} and to inhibit tyrosinase activity.⁷¹ Furthermore, as noted earlier, the presence of phenolic and flavonoids, whose amounts vary based on cocoon strain and extraction technique, may also augment Ser's antioxidant effects.¹⁸

Chronic low-grade inflammation, recognized as inflammaging, is a phenomenon closely linked to oxidative stress during aging. Oxidative stress triggers pro-inflammatory responses, while the resulting increase in cytokine levels aggravates oxidative stress, thereby forming a vicious cycle.^{72,73} This condition is implicated in age-associated vascular dysfunctions, such as ED.^{74,75} NF- κ B which can trigger inflammatory ED⁷⁶ is primarily activated under oxidative stress and translocates to the nucleus as p-NF- κ B, subsequently initiating the production of inflammatory cytokines.⁷⁷⁻⁷⁹ In our study, aged penile tissue showed increased p-NF- κ B/NF- κ B ratios and

elevated TNF- α and IL-6 levels. In this context, previous findings in aged rats indicated that levels of TNF- α and inflammatory mediators, such as C-reactive protein (CRP), were elevated not only in the circulation but also in cavernosal tissue, changes that adversely affected NO biosynthesis and erectile parameters.⁴⁴

Moreover, organ bath studies of cavernosal tissue from mice repeatedly infused with TNF- α showed that this cytokine impaired corpus cavernosum reactivity, evidently by altering endothelial and neuronal activity and reducing eNOS and nNOS expression.⁸⁰ Furthermore, Myung et al. demonstrated that IL-6 shifted corpus cavernosum reactivity toward contraction and reduced NO-mediated relaxation.⁸¹

Our results also showed that administration of Ser could normalize the p-NF- κ B/NF- κ B p65 ratio and reduced TNF- α and IL-6 concentrations. The anti-inflammatory effects of Ser may be in part mediated via the modulation of NF- κ B and the inhibition of inflammatory mediators, such as cyclooxygenase-2 and pro-inflammatory cytokines.^{17,22,82} Consistent with this rationale, Ser has been frequently used as an effective natural molecule for the attenuation of conditions including depression¹⁷, aging-induced liver damage²², osteoarthritis⁶⁹, and aging-induced cognitive impairments²¹, all of which are closely linked with inflammatory responses.

Previous studies have shown that attenuation of inflammatory responses is associated with restored endothelial function, as reflected by increased NOS expression, as well as enhanced cavernosal reactivity and ameliorated erectile parameters in aged and diabetic rats.^{44,83} Moreover, it has been shown that the knockout of the TNF- α gene in mice resulted in elevated cavernosal expression of eNOS and nNOS and increased erection frequency.⁸⁴ Furthermore, Sturny et al. reported that reduced penile expression of IL-6 and CRP was associated with the recovery of erectile function in a rat model of cavernous nerve injury.⁸⁵ Together, these findings suggest that modulating inflammatory signaling may exert beneficial effects against aging-induced alterations, further highlighting the therapeutic potential of anti-inflammatory compounds in the management of sexual dysfunction. This study has some limitations. The absence of a positive comparator group, specifically PDE5Is, represents a potential limitation of our study. It should be noted that the primary objective of the present study was to evaluate the efficacy and underlying mechanisms of Ser in improving sexual function in naturally aged rats, rather than to compare its effects with conventional pharmacological treatments. Nevertheless, the inclusion of a positive comparator would have provided an additional pharmacological framework for assessing the magnitude of the observed effects. Moreover, the findings should be interpreted according to the pre-specified biological domains, with the secondary biochemical, inflammatory, hormonal, and signaling findings providing supportive and mechanistic evidence rather than independent confirmatory outcomes. Additionally, while the biological replicates used in this study met the established standards of the field, the relatively small number of molecular and biochemical subsamples may have limited the statistical power of these analyses.

Conclusion

The findings of this study indicate that impaired copulatory behavior in aged rats is improved by Ser administration. This effect may be partially mediated through the elevation of serum testosterone levels, upregulation of the PI3K/Akt/eNOS axis, and activation of NO-cGMP signaling, driven in part by its antioxidant and anti-inflammatory properties. However, given the preclinical nature of this study, direct translation to clinical practice remains premature. Future researches must address dosing feasibility, pharmacokinetic profiles, and clinical assessments. In addition, subsequent animal studies should incorporate direct intracavernosal pressure measurements to fully validate functional hemodynamic recovery.

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Ethical Approval

The study received approval from the Ethics Committee of Tabriz University of Medical Sciences (Approval code: IR.TBZMED.AEC.1403.053).

Data Availability Statement

The team of authors is committed to ensuring that the data supporting our findings in this research will be made available to interested parties upon reasonable request. The requested data are available by contacting the corresponding authors.

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